| (51) International Patent Classification [7] : | | (11) International Publication Number: | WO 00/02996 |
| C12N | A2 | (43) International Publication Date: | 20 January 2000 (20.01.00) |

(21) International Application Number: PCT/US99/15425

(22) International Filing Date: 9 July 1999 (09.07.99)

(30) Priority Data:
60/092,357    10 July 1998 (10.07.98)    US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
   Without international search report and to be republished upon receipt of that report.

(54) Title: RECOMBINANT CONSTRUCTS AND SYSTEMS FOR SECRETION OF PROTEINS VIA TYPE III SECRETION SYSTEMS

(57) Abstract

   One aspect of the present invention relates to a DNA construct that contains a first DNA molecule encoding a functional type III secretion system, a promoter, and a second DNA molecule encoding a protein or polypeptide capable of being secreted by the type III secretion system. The second DNA molecule is operably coupled to the promoter so that upon introduction of the DNA construct into a host cell, the encoded protein or polypeptide and the type III secretion system are expressed and the encoded protein or polypeptide is secreted. Another aspect of the present invention relates to a system that includes (i) a first DNA construct having a first DNA molecule encoding a functional type III secretion system and (ii) a second DNA construct having a promoter operably coupled to a second DNA molecule encoding a protein or polypeptide capable of being secreted by the type III secretion system. Upon introduction of the first and second DNA constructs into a host cell, the encoded protein or polypeptide and the type III secretion system are expressed and the encoded protein or polypeptide is secreted. Methods of isolating a protein or polypeptide and identifying a gene encoding a potential effector protein or polypeptide are also disclosed.

# RECOMBINANT CONSTRUCTS AND SYSTEMS FOR SECRETION OF PROTEINS VIA TYPE III SECRETION SYSTEMS

This application claims the benefit of U.S. Provisional Patent Application Serial No. 60/092,357, filed July 10, 1998.

## BACKGROUND OF THE INVENTION

The most common bacterial pathogens of plants colonize the apoplast, and from that location outside of the walls of living cells they incite a variety of diseases in most cultivated plants (Alfano et al., "Bacterial Pathogens in Plants: Life Up Against the Wall," Plant Cell 8:1683-1698 (1996)). The majority of these are Gram-negative bacteria in the genera Erwinia, Pseudomonas, Xanthomonas, and Ralstonia. Most are host specific and will elicit the hypersensitive response ("HR") in nonhosts. The HR is a rapid, programmed death of plant cells in contact with the pathogen. Some of the defense responses associated with the HR are localized at the periphery of plant cells at the site of bacterial contact, but what actually stops bacterial growth is not known (Brown et al., "hrp genes in Xanthomonas campestris pv. vesicatoria Determine Ability to Suppress Papilla Deposition in Pepper Mesophyll Cells," MPMI 8:825-836 (1995); Young et al., "Changes in the Plasma Membrane Distribution of Rice Phospholipase D During Resistant Interactions With Xanthomonas oryzae pv. oryzae," Plant Cell 8:1079-1090 (1996); Bestwick et al., "Localization of Hydrogen Peroxide Accumulation During the Hypersensitive Reaction of Lettuce Cells to Pseudomonas syringae pv. phaseolicola," Plant Cell 9:209-221 (1997)). Pathogenesis in host plants, in contrast, involves prolonged bacterial multiplication, spread to surrounding tissues, and the eventual production of macroscopic symptoms characteristic of the disease. Although these bacteria are diverse in their taxonomy and pathology, they all possess hrp genes which direct their ability to elicit the HR in nonhosts or to be pathogenic (and parasitic) in hosts (Lindgren, "The Role of hrp Genes During Plant-Bacterial Interactions," Annu. Rev. Phytopathol. 35:129-152 (1997)). The hrp genes encode a type III protein secretion

system that appears to be capable of delivering Avr (avirulence) proteins across the walls
and plasma membranes of living plant cells (Alfano et al., "The Type III (Hrp) Secretion
Pathway of Plant Pathogenic Bacteria: Trafficking Harpins, Avr Proteins, and Death," J.
Bacteriol. 179:5655-5662 (1997), which is hereby incorporated by reference). The Avr
5      proteins are so named because they can betray the parasite to the R gene-encoded
surveillance system of plants, thereby triggering the HR (Vivian et al., "Avirulence
Genes in Plant-Pathogenic Bacteria: Signals or Weapons?," Microbiology 143:693-704
(1997); Leach et al., "Bacterial Avirulence Genes," Annul. Rev. Phytopathol. 34:153-
179 (1996)). But Avr-like proteins also appear to be key to parasitism in compatible host
10     plants, where the parasite proteins are undetected and the HR is not triggered. Thus,
bacterial avirulence and pathogenicity are interrelated phenomena and explorations of
HR elicitation are furthering our understanding of parasitic mechanisms.

Despite the emerging importance of Avr proteins, there is no direct
evidence that they travel the Hrp pathway, there is no knowledge of their function in
15     virulence, it appears likely that only a subset of those that are produced by typical host-
specific pathogens have been identified, and there is no evidence that they are produced
at all by host-promiscuous pathogens. The evidence that Avr proteins are transferred by
the Hrp pathway into plants is most complete, although still indirect, with *Pseudomonas
syringae* AvrB and AvrPto proteins. Nonpathogenic *Escherichia coli* and *Pseudomonas
20     fluorescens* cells that harbor the functional cluster of *Pseudomonas syringae hrp* genes
carried on cosmid pHIR11 can elicit an HR that is dependent on both the type III
secretion system and either AvrB or AvrPto (Gopalan et al., "Expression of the
*Pseudomonas Syringae* Avirulence Protein AvrB in Plant Cells Alleviates its
Dependence on the Hypersensitive Response and pathogenicity (Hrp) Secretion System
25     in Eliciting Genotype-specific Hypersensitive Cell Death," Plant Cell 8:1095-1105
(1996); Pirhonen et al., "Phenotypic Expression of *Pseudomonas Syringae avr* Genes in
*E. Coli* is Linked to the Activities of the *hrp*-encoded Secretion System," MPMI 9:252-
260 (1996)). Both Avr proteins trigger an R gene-dependent HR when transiently
expressed inside plant cells (Gopalan et al., "Expression of the *Pseudomonas Syringae
30     Avirulence Protein AvrB in Plant Cells Alleviates its Dependence on the Hypersensitive
Response and pathogenicity (Hrp) Secretion System in Eliciting Genotype-specific
Hypersensitive Cell Death," Plant Cell 8:1095-1105 (1996)) and the interaction of
AvrPto and Pto in the yeast two-hybrid system correlates with biological activity (Tang

et al., Science 274:2060 (1996); Scofield et al., Science 274:2063-2065 (1996)).

However, neither *Pseudomonas syringae, Escherichia coli* (pHIR11), nor

*Pseudomonas fluorescens* (pHIR11) secrete AvrB or AvrPto in culture, presumably

because these proteins travel the type III pathway directly into host cells and only upon

5    host cell contact, as with the Yop virulence proteins of *Yersinia* spp. (Gopalan et al.,

"Expression of the *Pseudomonas syringae* Avirulence Protein AvrB in Plant Cells

Alleviates its Dependence on the Hypersensitive Response and Pathogenicity (Hrp)

Secretion System in Eliciting Genotype-specific Hypersensitive Cell Death," Plant Cell

8:1095-1105 (1996); Cornelis et al., "The *Yersinia* Yop Regulon:  A Bacterial System for

10   Subverting Eukaryotic Cells," Mol. Microbiol. 23:861-867 (1997)).  Other known Avr

proteins have been observed only in the bacterial cytoplasm (Leach et al., "Bacterial

Avirulence Genes," Annu. Rev. Phytopathol. 34:153-179 (1996); Knoop et al.,

"Expression of the Avirulence Gene avrBs3 from *Xanthomonas campestris* pv.

*vesicatoria* is not Under the Control of hrp Genes and is Independent of Plant Factors," J.

15   Bacteriol. 173:7142-7150 (1991); Puri et al., "Expression of *avrPphB*, an Avirulence

Gene from *Pseudomonas Syringae* pv. *Phaseolicola*, and the Delivery of Signals

Causing the Hypersensitive Reaction in Bean," MPMI 10:247-256 (1997)).

Many proteins and polypeptides, including hormones and enzymes, are in

high demand for pharmacological and industrial use.  Once the gene encoding a desired

20   protein or polypeptide has been isolated, the protein can be produced readily through

fermentation in rapidly growing bacteria.  *Escherichia coli* is used most commonly for

large-scale protein production.  Current technology enables the production of relatively

large intracellular concentrations of the desired proteins or polypeptides.  Extraction of

the desired protein or polypeptide from the bacterial cells requires lysing of the cell

25   membrane.  After lysing the cell membrane, the desired protein or polypeptide is

contaminated with other proteins and, therefore, subject to degradation.  The resulting

contamination requires significant purification to obtain the isolated protein or

polypeptide and degradation of the desired protein or polypeptide limits the obtainable

yield.

30          In addition to fermentation technologies for production of proteins or

polypeptides, gene therapy involving transgenic plants is emerging as an important tool

for enhancing agricultural productivity and reducing disease losses.  For example,

transgenic plants expressing bacterial and viral proteins are now used for herbicide

tolerance and resistance to viral diseases, respectively. Because of the ease with which foreign proteins can be expressed in most major crops, it is feasible to bioprospect for proteins that will alter plant metabolism to enhance productivity and prevent losses due to pests.

5          Phytopathogenic bacteria contain a reservoir of genes encoding proteins that have evolved to be biologically active inside plants. Although poorly understood at this point, these proteins are likely to alter plant growth and development, affect fundamental cellular processes common to all higher organisms, including both plants and animals, and/or interact with defense mechanisms. The reservoir of these genes is

10        potentially large, but only a relatively small number have been identified among all of the phytopathogenic bacteria, because identifying them has been dependent upon inefficient procedures involving transgenic pathogens, plant inoculations, and plant reactions.

          Thus, it would be beneficial to obtain a recombinant construct and

15        expression system which overcomes these and other deficiencies in the art, particularly the ability to produce a recombinant host organism capable of expressing and secreting Avr and/or other desired proteins or polypeptides into their environment (i.e., culture medium).


20        **SUMMARY OF THE INVENTION**


          One aspect of the present invention relates to a DNA construct that contains a first DNA molecule encoding a functional type III secretion system, a promoter, and a second DNA molecule encoding a protein or polypeptide capable of

25        being secreted by the type III secretion system. The second DNA molecule is operably coupled to the promoter so that upon introduction of the DNA construct into a host cell, the encoded protein or polypeptide and the type III secretion system are expressed and the encoded protein or polypeptide is secreted. Also disclosed are host cells and expression systems that contain the DNA construct, as well as a method of secreting a

30        protein or polypeptide into the environment of a host cell which employs the DNA construct.

          Another aspect of the present invention relates to a system that includes a (i) first DNA construct having a first DNA molecule encoding a functional type III

secretion system and (ii) a second DNA construct having a promoter operably coupled to a second DNA molecule encoding a protein or polypeptide capable of being secreted by the type III secretion system. Upon introduction of the first and second DNA constructs into a host cell, the encoded protein or polypeptide and the type III secretion system are

5      expressed and the encoded protein or polypeptide is secreted. Also disclosed are host cells and expression systems that contain the system of DNA constructs, as well as a method of secreting a protein or polypeptide into the environment of a host cell which employs the system of DNA constructs.

A further aspect of the present invention relates to a method of isolating a

10     protein or polypeptide. This method is performed by providing a recombinant host cell that contains (i) a first DNA molecule encoding a functional type III secretion system and (ii) a second, heterologous DNA molecule having a promoter operably coupled to a nucleic acid sequence encoding a protein or polypeptide capable of being secreted by the type III secretion system. The recombinant host cell is introduced into a culture medium,

15     wherein the encoded protein or polypeptide and the type III secretion system are expressed and the encoded protein or polypeptide is secreted into the culture medium. Subsequently, the encoded protein or polypeptide is isolated from the culture medium.

Still another aspect of the present invention relates to a method of identifying a gene encoding a potential effector protein or polypeptide. This method of

20     the invention is performed by providing a host cell that contains a DNA molecule encoding a functional type III secretion system. Next, a candidate gene encoding a protein or polypeptide is inserted into the host cell under conditions effective to express the encoded protein or polypeptide. Finally, it is determined whether the encoded protein or polypeptide is secreted by the recombinant host cell, wherein secretion of the

25     encoded protein or polypeptide indicates that the gene encodes a potential effector protein or polypeptide.

Since the DNA constructs of the present invention enable expression and secretion of proteins by recombinant host cells, it is possible to employ these recombinant host cells in a fermentation system which enables efficient production of a

30     desired protein or polypeptide that can be purified at high yield and at minimal expense compared to existing fermentation/purification procedures. Moreover, the constructs of the present invention can be employed to bioprospect for potential effector proteins or polypeptides, which by virtue of their expression and secretion by a recombinant host

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cell expressing a type III secretion system, become likely candidates as effector proteins. This method of screening for potential effector protein is novel and much more systematic and efficient than prior methods.

5                            **BRIEF DESCRIPTION OF THE DRAWINGS**


                  Figure 1 is a diagram of the physical maps for cosmids pCPP2156 and pCPP2157, which contain the *Erwinia chrysanthemi hrp* region, and comparison of the *hrp* regions of *Erwinia chrysanthemi* and *Erwinia amylovora* (Bauer et al., "*Erwinia*
10      *chrysanthemi* harpin$_{Ech}$: An Elicitor of the Hypersensitive Response that Contributes to Soft-rot Pathogenesis," MPMI 8:484-491 (1995); Kim et al., "The hrpC and hrpN Operons of *Erwinia chrysanthemi* EC16 are Flanked by plcA and Homologs of Hemolysin/Adhesin Genes and Accompanying Activator/Transporter Genes," MPMI 11(6):563-567 (1998); Bogdanove et al., "*Erwinia amylovora* Secretes Harpin via a
15      Type III of Pathway and Contains a Homolog of YopN of *Yersinia* spp.," J. Bacteriol. 178:1720-1730 (1996); Wei et al., "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science, 257:85-88 (1992); Wei et al., "HrpI of *Erwinia amylovora* Functions in Secretion of Harpin and is a Member of a New Protein Family," J. Bacteriol., 175:7958-7967 (1993); Kim et al., "The HrpA and
20      HrpC Operons of *Erwinia amylovora* Encode Components of a Type III Pathway that Secrets Harpin," J. Bacteriol. 179:1690-1697 (1997), which are hereby incorporated by reference). Arrow-shaped boxes denote putative transcriptional units. Shadowed areas denote *hrp* regions. Dashed boxes denote transcriptional units predicted on the basis of the homology and spacing of partially sequenced regions (shaded areas) in comparison
25      with the corresponding *Erwinia amylovora hrp* genes. The filled triangle indicates the location of mini-Tn5*Cm* in pCPP2368.
                  Figure 2A contains images of *Nicotiana clevelandii* leaves infiltrated with *Escherichia coli* DH5α carrying *Erwinia chrysanthemi hrp* clusters that are either intact (pCPP2156, pCPP2416) or defective (pCPP2157, pCPP2368) at a concentration of
30      5 X 10$^8$ cfu/ml. Leaves were photographed 48 hours after infiltration. Tissue collapse occurred within 24 hours. The areas below each number on the leaf was infiltrated with *Escherichia coli* DH5α carrying following constructs: 1, pCPP2156; 2, pCPP2156 and

pAVRB-FLAG2; 3, pCPP2157; 4, PCPP2157 and pAVRB-FLAG2; 5, pCPP2416 and
pAVRB-FLAG2; 6, pCPP2368 and pAVRB-FLAG2.

        Figure 2B contains images of the leaves of two soybean cultivars,
Norchief (*RPG1*) and Acme (*rpg1*), infiltrated with *Escherichia coli* DH5α carrying

5    *Erwinia chrysanthemi hrp* clusters that are either intact (pCPP2156, pCPP2416) or
defective (pCPP2157, pCPP2368) at a concentration of 5 X $10^8$ cfu/ml. Leaves were
photographed 72 hours after infiltration. Tissue collapse occurred within 48 hours. The
first two leaves (numbers 1 through 4) are Norchief and the third leaf (number 5 and 6) is
Acme. The area below each number on the leaf was infiltrated with *Escherichia coli*

10   DH5α carrying following constructs: 1, pCPP2416; 2, pCPP2416 and pAVRB-FLAG2;
3, pCPP2368; 4, PCPP2368 and pAVRB-FLAG2; 5, pCPP2156; 6, pCPP2156 and
pAVRB-FLAG2.

        Figure 3 is an image of an immunodetection in western blots showing
differential secretion of AvrB-FLAG by *Escherichia coli* DH5α carrying either a wild

15   type (pCPP2156) or mutant (pCPP2368) *Erwinia chrysanthemi hrp* cluster. The
supernatant fraction (S) was concentrated 7.5 X more than the cell pellet fraction (P).
Lanes: 1, *Escherichia coli*(pCPP2156), pAVRB-FLAG2); 2, *Escherichia
coli*(pCPP2368, pAVRB-FLAG2); 3, *Escherichia coli*(pCPP2156, pAVRB-FLAG2); 4,
*Escherichia coli*(pCPP2368), pAVRB-FLAG2).

20        Figure 4 is an image of an immunodetection in western blots showing
differential secretion of AvrB by *Escherichia coli* DH5α carrying either a wild type
(pCPP2156) or mutant (pCPP2368) *Erwinia chrysanthemi hrp* cluster. pCPP2138
encodes mature ß-lactamase, which was used as a cytoplasmic marker. The supernatant
fraction (S) was concentrated 7.5 X more than the cell pellet fraction (P) for lanes 1 and

25   2 and 15 X more for lanes 6 and 7. Lanes: 1, *Escherichia coli*(pCPP2156, pAVRB1); 2,
*Escherichia coli*(pCPP2368, pAVRB1); 3, *Escherichia coli*(pCPP2156, pAVRB1); 4,
*Escherichia coli*(pCPP2368), pAVRB1); 5, purified AvrB; 6, *Escherichia
coli*(pCPP2156, pAVRB1, pCPP2318); 7, *Escherichia coli*(pCPP2368, pAVRB1,
pCPP2318); 8 *Escherichia coli*(pCPP2156, pAVRB1, pCPP2318); 9, *Escherichia

30   coli*(pCPP2368, pAVRB1, pCRPP2318).

        Figure 5 is an image of an immunodetection in western blots showing
differential secretion of AvrPto-FLAG by *Escherichia coli* DH5α carrying either intact
(pCPP2156) or defective (pCPP2157, pCPP2368) *Erwinia chrysanthemi hrp* clusters and

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*Escherichia coli* MC4100 carrying pHIR11. As indicated above, pCPP2138 encodes

mature ß-lactamase, which was used as a cytoplasmic marker. The supernatant fraction

(S) was concentrated 7.5 X more than the cell pellet fraction (P). Lanes: 1, *Escherichia*

*coli*(pCPP2156), pCPP2318, pAVRPTO-FLAG); 2, *Escherichia coli*(pCPP2157,

5    pCPP2318, pAVRPTO-FLAG); 3, *Escherichia coli*(pCPP2368, pCPP2318, pAVRPTO-

FLAG); 4, *Escherichia coli*(pCPP2156, pCPP2318, pAVRPTO-FLAG); 5, *Escherichia*

*coli*(pCPP2157, pCPP2318, pAVRPTO-FLAG); 6, *Escherichia coli*(pCPP2368,

pCPP2318, pAVRPTO-FLAG); 7, *Escherichia coli*(pHIR11, pAVRPTO-FLAG); 8,

*Escherichia coli*(pHIR11, pAVRPTO-FLAG).

10                Figure 6 is an image of the immunodetection of DspE and DspEÆ1521 in

western blots of culture supernatant and bacterial cell fractions using anti-DspE

antiserum (see Bogdanove et al., J. Bacteriol. 180:2244-2247 (1998), which is hereby

incorporated by reference). Lane 1, hrp mutant Ea273-K178; lane 2, wild-type strain

Ea273; lane 3, partial dspE deletion mutant Ea273dspEÆ1521. The migration of the

15   molecular weight markers (BRL) is indicated at the left.

Figure 7 is a diagram showing the genetic organization of the hrp and dsp

genes contained by cosmids pCPP430 and pCPP431. The letters designating the known

or proposed functions correspond to the following: S, secretion; R, regulation; H, harpin;

A, avirulence; D, disease; U, unknown.

20                Figure 8 is an image of an immunodetection in western blots of flagged

*Pseudomonas syringae* Avr proteins in cell (C) and supernatant (S) fractions from

cultures of *Escherichia coli* DH5 containing the designated Avr protein (AvrB-Flag or

AvrPto-Flag) and the hrp/dsp cluster (pCPP430), the minimal hrp cluster (pCPP431), or

the secretion-defective hrp cluster (pCPP430hrcVÐ).

25

## DETAILED DESCRIPTION OF THE INVENTION

One aspect of the present invention relates to novel constructs that can be

used to transform host cells so that they express and secrete (i.e., into the host cell

30   environment) a protein or polypeptide of interest.

According to one embodiment, a DNA construct is provided which

includes a first DNA molecule encoding a functional type III secretion system, a

promoter, and a second DNA molecule encoding a protein or polypeptide capable of

being secreted by the type III secretion system. The second DNA molecule is operably

coupled to the promoter so that upon introduction of the DNA construct into a host cell,

the encoded protein or polypeptide and the type III secretion system are expressed and

the encoded protein or polypeptide is secreted by the host cell.

5          According to a second embodiment, a pair of DNA constructs are utilized

as part of a system. The first DNA construct includes a DNA molecule encoding a

functional type III secretion system. The second DNA construct includes a promoter

operably coupled to a DNA molecule encoding a protein or polypeptide capable of being

secreted by the type III secretion system. Upon introduction of the first and second DNA

10         constructs into a host cell, the protein or polypeptide and the type III secretion system are

expressed and the protein or polypeptide is secreted.

           As used in each of the above embodiments, any functional type III

secretion system can be employed. By functional, it is intended that the type III

secretion system contain all required genes under appropriate transcriptional and/or

15         translational control such that the secretion system can secrete proteins or polypeptides

that are capable of being secreted. Preferred type III secretion systems are those

obtained from the genus *Erwinia*, more preferably the harpin secretion systems obtained

from *Erwinia amylovora* or *Erwinia chrysanthemi*, and *Pseudomonas*, more preferably

the harpin secretion systems obtained from *Pseudomonas syringae*. For example, the

20         harpin secretion system of *Erwinia amylovora* is present on cosmid pCPP430 (Beer et

al., "The *hrp* Gene Cluster of *Erwinia amylovora*," in Advances in Molecular Genetics

of Plant-Microbe Interactions, Proceedings of the 5th International Symposium on the

Molecular Genetics of Plant-Microbe Interactions, Interlaken, Switzerland, September,

1990, pp. 53-60 (1991) which is hereby incorporated by reference) and the harpin

25         secretion system of *Erwinia carotovora* is present in cosmid pCPP2156 (Ham et al., "A

Cloned *Erwinia chrysanthemi* Hrp (type III Protein Secretion) System Functions in

*Escherichia coli* to Deliver *Pseudomonas syringae* Avr Signals to Plant Cells and to

Secrete Avr Proteins in Culture," Proc. Natl. Acad. Sci. USA, 95(17): 10206-11 (1998),

which is hereby incorporated by reference). A diagram of cosmid pCPP430 is shown at

30         Figure 7 and a diagram of cosmid pCPP2156 is shown at Figure 1.

           Type III protein secretion systems are present in bacterial pathogens of

both animals and plants, and are typified by the type III system of *Yersinia* spp. (Finlay

et al., "Common Themes in Microbial Pathogenicity Revisited," Microbiol. Mol. Biol.

Rev., 61:136-169 (1997); Cornelis et al., "The *Yersinia* Yop Regulon: A Bacterial

System for Subverting Eukaryotic Cells," Mol. Microbiol., 23:861-867 (1997), which are

hereby incorporated by reference). These animal pathogens are primarily extracellular

parasites, and their Yops (*Yersinia* outer proteins) are secreted and translocated directly

5    into host cells in a contact-dependent manner (Cornelis et al., "The *Yersinia* Yop

Regulon: A Bacterial System for Subverting Eukaryotic Cells," Mol. Microbiol.,

23:861-867 (1997), which is hereby incorporated by reference). A similar host-contact

dependency may operate in most plant pathogenic bacteria. Nine of the *hrp* genes are

universal components of type III secretion systems, and these have been renamed *hrc*

10    (HR and conserved) and given the last-letter designation of their *Yersinia* homolog (with

the exception of *hrcV*) (Bogdanove et al., "Unified Nomenclature for Broadly Conserved

*hrp* Genes of Phytopathogenic Bacteria," Mol. Microbiol., 20:681-683 (1996), which is

hereby incorporated by reference). The Hrc proteins enable protein movement across the

bacterial inner and outer membranes independently of the general protein export (Sec)

15    pathway (Charkowski et al., "Altered Localization of HrpZ in *Pseudomonas syringae* pv.

*syringae hrp* Mutants Suggests That Different Components of the Type III Secretion

Pathway Control Protein Translocation Across the Inner and Outer Membranes of Gram-

negative Bacteria," J. Bacteriol., 179:3866-3874 (1997), which is hereby incorporated by

reference). In contrast to the Hrc proteins, the Hrp proteins may be peripheral

20    components of the Hrp secretion system and are more likely to perform type III secretion

functions that are extracellular and specific to protein transfer across the plant cell wall

and plasma membrane.

       The genes encoding type III secretion systems are usually clustered, and

the emerging concept that genes with related functions in virulence are often grouped on

25    plasmids or in horizontally-acquired pathogenicity islands has important implications

throughout pathogenic microbiology (Lawrence et al., "Selfish Operons: Horizontal

Transfer May Drive the Evolution of Gene Clusters," Genetics, 143:1843-1860 (1996);

Groisman et al., "Pathogenicity Islands: Bacterial Evolution in Quantum Leaps," Cell,

87:791-794 (1996); Hacker et al., "Pathogenicity Islands of Virulent Bacteria: Structure,

30    Function and Impact on Microbial Evolution," Mol. Microbiol., 23:1089-1097 (1997),

which are hereby incorporated by reference). There is some evidence for horizontal

acquisition of *hrp* gene clusters in plant pathogenic bacteria, and the *hrp* cluster in

*Ralstonia solanacearum* is carried on a megaplasmid (Alfano et al., "Bacterial Pathogens

in Plants: Life Up Against the Wall," <u>Plant Cell</u>, 8:1683-1698 (1996), which is hereby incorporated by reference). The finding of a plasmid-borne *hrp* gene cluster in *Erwinia herbicola* pv. *gypsophilae* suggests that virulence may be acquired readily by plant-associated bacteria (Nizan et al., "The Presence of *hrp* Genes on the Pathogenicity-

5    associated Plasmid of the Tumorigenic Bacterium *Erwinia herbicola* pv. *gypsophilae*," <u>MPMI</u>, 10:677-682 (1997), which is hereby incorporated by reference). *Erwinia herbicola* is a common epiphyte that is usually benign, but strains classified as *Erwinia herbicola* pv. *gypsophilae* cause galls on gypsophila and elicit the HR in tobacco. A 150-kb plasmid carries phytohormone biosynthetic genes and *hrp* genes, and the latter

10   are required both for gall formation and HR elicitation (Nizan et al., "The presence of *hrp* genes on the pathogenicity-associated Plasmid of the Tumorigenic Bacterium *Erwinia herbicola* pv. *gypsophilae*," <u>MPMI</u>, 10:677-682 (1997), which is hereby incorporated by reference).

             The clustering of genes with related function is also consistent with the

15   ability of some cloned *hrp* clusters to enable nonpathogens like *Escherichia coli* to elicit the HR. This has been reported for cosmids pHIR11 from *Pseudomonas syringae* pv. *syringae*, pCPP430 from *Erwinia amylovora*, pPPY430 from *Pseudomonas syringae* pv. *phaseolicola*, and pCPP2156 from *Erwinia chrysanthemi* (Alfano et al., "Bacterial Pathogens in Plants: Life Up Against the Wall," <u>Plant Cell</u>, 8:1683-1698 (1996); Puri et

20   al., "Expression of *avrPphB*, an Avirulence Gene from *Pseudomonas syringae* pv. *phaseolicola*, and the Delivery of Signals Causing the Hypersensitive Reaction in Bean," <u>MPMI</u>, 10:247-256 (1997); Ham et al., "A Cloned *Erwinia chrysanthemi* Hrp (type III Protein Secretion) System Functions in *Escherichia coli* to Deliver *Pseudomonas syringae* Avr Signals to Plant Cells and to Secrete Avr Proteins in Culture," <u>Proc. Natl.</u>

25   <u>Acad. Sci. USA</u>, 95(17): 10206-11 (1998), which are hereby incorporated by reference). Although these cosmids support heterologous HR elicitation, they do not enable *Escherichia coli* to become pathogenic. The basis for HR elicitation is best understood with pHIR11. The cosmid carries a 25-kb set of *hrp* genes that is intact and functional, as revealed by DNA sequencing and the ability to direct secretion of the HrpZ harpin

30   (Alfano et al., "The Type III (Hrp) Secretion Pathway of Plant Pathogenic Bacteria: Trafficking Harpins, Avr Proteins, and Death," <u>J. Bacteriol.</u> 179:5655-5662 (1997), which is hereby incorporated by reference). The cosmid also carries, adjacent to the *hrp* cluster, the *hrmA* gene, which is *avr*-like in producing an avirulence phenotype when

expressed in a tobacco pathogen and in being lethal when heterologously expressed inside nonhost tobacco cells (Alfano et al., "Evidence That the *Pseudomonas syringae* pv. *syringae hrp*-linked *hrmA* Gene Encodes an Avr-like Protein that Acts in a *hrp*-dependent Manner Within Tobacco Cells," MPMI, 10:580-588 (1997), which is hereby

5      incorporated by reference). The concept that the minimal requirement for bacterial elicitation of the HR is a functional Hrp system and an *avr* gene whose product is recognized by the *R*-gene surveillance system of the test plant is supported by experiments in which the HR is observed only when an appropriate, heterologous *avr* gene is supplied in trans of the *hrp*⁺ cosmid (Puri et al., "Expression of *avrPphB*, an

10     Avirulence Gene from *Pseudomonas syringae* pv. *phaseolicola*, and the Delivery of Signals Causing the Hypersensitive Reaction in Bean," MPMI, 10:247-256 (1997); Ham et al., "A Cloned *Erwinia chrysanthemi* Hrp (type III Protein Secretion) System Functions in *Escherichia coli* to Deliver *Pseudomonas syringae* Avr Signals to Plant Cells and to Secrete Avr Proteins in Culture," Proc. Natl. Acad. Sci. USA, 95(17):

15     10206-11 (1998); Gopalan, S. et al., "Expression of the *Pseudomonas syringae* Avirulence Protein AvrB in Plant Cells Alleviates its Dependence on the Hypersensitive Response and pathogenicity (Hrp) Secretion System in Eliciting Genotype-specific Hypersensitive Cell Death," Plant Cell, 8:1095-1105 (1996); Pirhonen et al., "Phenotypic Expression of *Pseudomonas syringae avr* Genes in *E. Coli* is Linked to the Activities of

20     the *hrp*-encoded Secretion System," MPMI 9:252-260 (1996), which are hereby incorporated by reference).

            *hrp* genes are expressed in plants and in apoplast-mimicking minimal media, but typically not in complex media (Lindgren, "The Role of *hrp* Genes During Plant-Bacterial Interactions," Annu. Rev. Phytopathol, 35:129-152 (1997), which is

25     hereby incorporated by reference). The Hrp regulatory systems in plant pathogenic bacteria can be divided into two groups, which correspond also to differences in *hrp* cluster composition (Alfano et al., "The Type III (Hrp) Secretion Pathway of Plant Pathogenic Bacteria: Trafficking Harpins, Avr Proteins, and Death," J. Bacteriol. 179:5655-5662 (1997), which is hereby incorporated by reference). In the group I Hrp

30     systems of *Erwinia* and *Pseudomonas*, *hrp* operons are activated by HrpL, an alternate sigma factor (Lindgren, "The Role of *hrp* Genes During Plant-Bacterial Interactions," Annu. Rev. Phytopathol. 35:129-152 (1997); Hutcheson, "The *hrp*-encoded Protein Export Systems of *Pseudomonas syringae* and Other Plant Pathogenic Bacteria and Their

Role in Pathogenicity," in Plant-Microbe Interactions, Volume 3, ed. Stacey and Keen,

New York: Chapman and Hall, Inc., pp. 145-179 (1997), which are hereby incorporated

by reference). In contrast, *hrp* transcription is activated by an AraC homolog in the

group II Hrp systems of *Xanthomonas* (HrpX) and *Ralstonia* (HrpB) (Lindgren, "The

5      Role of *hrp* Genes During Plant-Bacterial Interactions," Annu. Rev. Phytopathol.

35:129-152 (1997), which is hereby incorporated by reference). Upstream activators of

these factors have been described for *Pseudomonas syringae* (HrpR and HrpS, $\Phi^{54}$-

dependent promoter enhancer-binding protein homologs) (Lindgren, "The Role of *hrp*

Genes During Plant-Bacterial Interactions", Annu. Rev. Phytopathol, 35:129-152 (1997);

10     Hutcheson, "The *hrp*-encoded Protein Export Systems of *Pseudomonas syringae* and

Other Plant Pathogenic Bacteria and Their Role in Pathogenicity," in Plant-Microbe

Interactions, Volume 3, ed. Stacey and Keen, New York: Chapman and Hall, Inc., pp.

145-179 (1997), which are hereby incorporated by reference), *Xanthomonas campestris*

pv. *vesicatoria* (HrpG, OmpR homolog) (Wengelnik et al., "HrpG, a Key *hrp* Regulatory

15     Protein of *Xanthomonas campestris* pv. *vesicatoria* is Homologous to Two-component

Response Regulators," MPMI 9:704-712 (1996), which is hereby incorporated by

reference), and *R. solanacearum* (PrhA) (Marenda et al., "PrhA Controls a Novel

Regulatory Pathway Required for the Specific Induction of *Ralstonia solanacearum hrp*

Genes in the Presence of Plant Cells," Mol. Microbiol. 27:437-453 (1998), which is

20     hereby incorporated by reference). The recent discovery of PrhA is particularly

significant because this homolog of TonB-dependent outer membrane siderophore

receptors, which appears to act at the top of the Hrp regulatory hierarchy, is required

specifically for induction of *hrp* genes in the presence of plant cells and for full virulence

in Arabidopsis (Marenda et al., "PrhA Controls a Novel Regulatory Pathway Required

25     for the Specific Induction of *Ralstonia solanacearum hrp* Genes in the Presence of Plant

Cells," Mol. Microbiol. 27:437-453 (1998), which is hereby incorporated by reference).

In the host-promiscuous pathogen *Erwinia carotovora*, production of the *hrpN*-encoded

harpin is activated by the quorum sensing signal, *N*-(3-oxohexanoyl)-L-homoserine

lactone and negatively regulated by RsmA, two global regulators similarly controlling

30     exoenzyme production (Mukherjee et al., "Molecular Characterization and Expression of

the *Erwinia carotovora hrpN*$_{Ecc}$ gene, Which Encodes An Elicitor of the Hypersensitive

Reaction," MPMI 10:462-471 (1997); Cui, Y. et al., "The RsmA⁻ Mutants of *Erwinia*

*carotovora* subsp. *carotovora* Strain Ecc71 Overexpress hrpN$_{Ecc}$ and Elicit a

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Hypersensitive Reaction-like Response in Tobacco Leaves," MPMI, 9-6565-573 (1996),
which are hereby incorporated by reference).

        The protein or polypeptide must be compatible for secretion by the type
III secretion system employed. By compatible, it is intended that the protein or
polypeptide contain a secretion signal that can be recognized by the particular type III
secretion system that is employed. The secretion signal enables the expressed protein or
polypeptide to be recognized by the type III secretion system and transported via the
expressed secretion system into the extracellular environment in which the recombinant
host cells exist, i.e., culture medium.

        Suitable secretion signals can be either an mRNA or a polypeptide
fragment of a naturally-occurring protein secreted by the type III secretion system.
Compatible secretion signals can readily be determined for any particular
type III secretion system that is to be employed. By identifying proteins that are
normally secreted by the type III secretion system, it is possible to prepare deletion
mutants missing various fragments of the full length protein that is normally secreted by
the secretion system. Using labeled antibodies raised against epitopes of the various
deletion fragments that are expressed (i.e., N-terminal epitopes, C-terminal epitopes,
etc.), it is possible to identify deletion mutants that are secreted and those that are not
secreted. Thus, protein domains necessary for secretion of the full length protein can be
readily identified. Once the protein domains have been identified and sequenced, they
can be utilized as secretion signals in fusion proteins of the present invention.

        Typically, the secretion signal is an N-terminal domain of a protein that is
normally secreted by the particular type III secretion system. An exemplary secretion
signal is a 201 amino acid sequence from the N-terminal domain of the DspE protein of
*Erwinia amylovora*, which has an amino acid sequence corresponding to SEQ. ID. No. 1
as follows:

```
Met Glu Leu Lys Ser Leu Gly Thr Glu His Lys Ala Ala Val His Thr
1             5                 10              15

Ala Ala His Asn Pro Val Gly His Gly Val Ala Leu Gln Gln Gly Ser
            20              25                  30

Ser Ser Ser Ser Pro Gln Asn Ala Ala Ala Ser Leu Ala Ala Glu Gly
        35              40                  45

Lys Asn Arg Gly Lys Met Pro Arg Ile His Gln Pro Ser Thr Ala Ala
        50              55              60
```

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```
Asp Gly Ile Ser Ala Ala His Gln Gln Lys Lys Ser Phe Ser Leu Arg
65              70              75              80

Gly Cys Leu Gly Thr Lys Lys Phe Ser Arg Ser Ala Pro Gln Gly Gln
              85              90              95

Pro Gly Thr Thr His Ser Lys Gly Ala Thr Leu Arg Asp Leu Leu Ala
          100             105             110

Arg Asp Asp Gly Glu Thr Gln His Glu Ala Ala Ala Pro Asp Ala Ala
          115             120             125

Arg Leu Thr Arg Ser Gly Gly Val Lys Arg Arg Asn Met Asp Asp Met
      130             135             140

Ala Gly Arg Pro Met Val Lys Gly Gly Ser Gly Glu Asp Lys Val Pro
145             150             155             160

Thr Gln Gln Lys Arg His Gln Leu Asn Asn Phe Gly Gln Met Arg Gln
              165             170             175

Thr Met Leu Ser Lys Met Ala His Pro Ala Ser Ala Asn Ala Gly Asp
          180             185             190

Arg Leu Gln His Ser Pro Pro His Ile
          195             200
```

This amino acid sequence is encoded by a DNA molecule having a 603 base nucleic acid sequence from the gene or gene fragment coding for DspE. This DNA molecule has a nucleotide sequence corresponding to SEQ. ID. No. 2 as follows:

```
ATGGAATTAA AATCACTGGG AACTGAACAC AAGGCGGCAG TACACACAGC GGCGCACAAC    60
CCTGTGGGGC ATGGTGTTGC CTTACAGCAG GGCAGCAGCA GCAGCAGCCC GCAAAATGCC   120
GCTGCATCAT TGGCGGCAGA AGGCAAAAAT CGTGGGAAAA TGCCGAGAAT TCACCAGCCA   180
TCTACTGCGG CTGATGGTAT CAGCGCTGCT CACCAGCAAA AGAAATCCTT CAGTCTCAGG   240
GGCTGTTTGG GGACGAAAAA ATTTTCCAGA TCGGCACCGC AGGGCCAGCC AGGTACCACC   300
CACAGCAAAG GGGCAACATT GCGCGATCTG CTGGCGCGGG ACGACGGCGA AACGCAGCAT   360
GAGGCGGCCG CGCCAGATGC GGCGCGTTTG ACCCGTTCGG GCGGCGTCAA ACGCCGCAAT   420
ATGGACGACA TGGCCGGGCG GCCAATGGTG AAAGGTGGCA GCGGCGAAGA TAAGGTACCA   480
ACGCAGCAAA AACGGCATCA GCTGAACAAT TTTGGCCAGA TGCGCCAAAC GATGTTGAGC   540
AAAATGGCTC ACCCGGCTTC AGCCAACGCC GGCGATCGCC TGCAGCATTC ACCGCCGCAC   600
ATC                                                               603
```

The 201 amino acid secretion signal of *Erwinia amylovora* DspE is compatible with the harpin secretion system of *Erwinia amylovora*. Other secretion signals that are compatible with various type III secretion systems have been described in the art and other are continually being identified.

5              Human-pathogenic *Yersinia* spp. use the type III secretion system to deliver seven effector Yops into the host cytoplasm and another three Yops to the bacterial milieu (Hueck, "Type III Protein Secretion Systems in Bacterial Pathogens of Animals and Plants," Microbiol. Mol. Biol. Rev. 62:379-433 (1998), which is hereby incorporated by reference). All Yops carry an mRNA targeting signal in their first 15

10 codons (Anderson and Schneewind, "A mRNA Signal for the Type III Secretion of Yop Proteins by *Yersinia enterocolitica*," Science 278:1140-1143 (1997); Anderson and Schneewind, "Type III Machines of Gram-negative Pathogens: Injecting Virulence Factors into Host Cells and More," Curr. Opin. Microbiol. 2:18-24 (1999), which are hereby incorporated by reference). Fusion of the first 15 codons of YopE to an Npt

15 reporter is sufficient for type III secretion of the hybrid to the bacterial milieu, and mutations shifting the reading frame of these codons do not abolish secretion (Anderson and Schneewind, "A mRNA Signal for the Type III Secretion of Yop Proteins by *Yersinia enterocolitica*", Science 278:1140-1143 (1997), which is hereby incorporated by reference). Thus, the targeting information resides in the mRNA rather than the

20 encoded peptide. The mRNA targeting signal appears universal among effector proteins secreted by the type III pathways of animal pathogens and plant pathogens. For example, *Yersinia enterocolitica* strongly secretes AvrB and AvrPto, and *Escherichia coli*(pCPP2156) secretes (much less efficiently) YopE and YopQ (Anderson et al., "mRNA Signal Universal to Plant and Animal Pathogens," Proc. Natl. Acad. Sci. USA

25 (1999), which is hereby incorporated by reference). The first 15 codons of *avrB* and *avrPto* are necessary for secretion from *Escherichia coli*(pCPP2156), and they are sufficient to target an AvrPto$_{1-15}$-Npt hybrid for secretion in *Yersinia* and *Pseudomonas syringae* (Anderson et al., "mRNA Signal Universal to Plant and Animal Pathogens," Proc. Natl. Acad. Sci. USA (1999), which is hereby incorporated by reference). Frame-

30 shift mutations changing the peptide encoded by the AvrPto$_{1-15}$ mRNA do not prevent secretion of Npt in *Yersinia* (Anderson et al., "mRNA Signal Universal to Plant and Animal Pathogens," Proc. Natl. Acad. Sci. USA (1999), which is hereby incorporated by

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reference). Thus, the mRNA signal recognized by type III secretion systems appears to be universal.

    The protein or polypeptide can be a naturally secreted protein or polypeptide homologous to the type III secretion system (i.e., normally secreted by the

5 source organism from which the type III secretion system was obtained) or heterologous to the type III secretion system (i.e., normally secreted by a source organism other than that from which the type III secretion system was obtained). By way of example, a naturally secreted protein or polypeptide homologous to the harpin secretion systems of *Erwinia amylovora* include, among others, DspE and HrpN. Exemplary naturally

10 secreted proteins or polypeptides which are heterologous to the harpin secretion systems of *Erwinia* include the various *Pseudomonas syringae* Avr proteins.

    Two classes of extracellular Hrp proteins have now been defined – harpins and pilins. Harpins are glycine-rich proteins that lack cysteine, are secreted in culture when the Hrp systems is expressed, and possess heat-stable HR elicitor activity

15 when infiltrated into the leaves of tobacco and several other plants (Alfano et al., "Bacterial Pathogens in Plants: Life Up Against the Wall," Plant Cell, 8:1683-1698 (1996), which is hereby incorporated by reference). Mutation of the prototypical *hrpN* harpin gene in *Erwinia amylovora* Ea321 strongly diminishes HR and pathogenicity phenotypes (Kim et al., "HrpW of *Erwinia amylovora*, a New Harpin That is a Member

20 of a Proposed Class of Pectate Lyases," J. Bacteriol. 180(19):5203-5210 (1998), which is hereby incorporated by reference), but mutation of the *hrpZ* harpin gene in different *Pseudomonas syringae* strains has little or no effect on Hrp phenotypes (Alfano et al., "Analysis of the Role of the *Pseudomonas syringae* pv. *syringae* HrpZ Harpin in Elicitation of the Hypersensitive Response in Tobacco Using Functionally Nonpolar

25 Deletion Mutations, Truncated HrpZ Fragments, and *hrmA* Mutations," Mol. Microbiol. 19:715-728 (1996); Charkowski et al., "The *Pseudomonas syringae pv. tomato* HrpW Protein Has Domains Similar to Harpins and Pectate Lyases and Can Elicit the Plant Hypersensitive Response and Bind to Pectate," J. Bacteriol. 180 (19):5211-5217 (1998), which are hereby incorporated by reference). The natural function of harpins or the basis

30 for their ability to elicit an apparent programmed cell death when artificially introduced into the apoplast of plants is unknown. However, two lines of evidence point to a site of action in the plant cell wall. First, purified *Pseudomonas syringae* harpin binds to cell walls and has biological activity only with walled cells (Hoyos et al., "The Interaction of

Harpin$_{Pss}$ With Plant Cell Walls," MPMI 9:608-616 (1996), which is hereby incorporated

by reference). Second, HrpW, a second harpin discovered in both *Erwinia amylovora*

and *Pseudomonas syringae*, has an N-terminal half that is harpin-like but a C-terminal

half that is homologous to a newly-defined class of pectate lyases found in fungal and

5       bacterial pathogens (Kim et al., "HrpW of *Erwinia amylovora*, a New Harpin That is a

Member of a Proposed Class of Pectate Lyases," J. Bacteriol. 180(19):5203-5210 (1998);

Charkowski et al., "The *Pseudomonas syringae pv. tomato* HrpW Protein Has Domains

Similar to Harpins and Pectate Lyases and Can Elicit the Plant Hypersensitive Response

and Bind to Pectate," J. Bacteriol. 180 (19):5211-5217 (1998), which are hereby

10      incorporated by reference). Elicitor activity resides in the harpin domain, and the pectate

lyase domain, although lacking enzymatic activity, binds specifically to pectate

(Charkowski, A. et al., "The *Pseudomonas syringae pv. tomato* HrpW Protein Has

Domains Similar to Harpins and Pectate Lyases and Can Elicit the Plant Hypersensitive

Response and Bind to Pectate," J. Bacteriol. 180 (19):5211-5217 (1998), which is hereby

15      incorporated by reference). The second class of extracellular Hrp proteins are

represented by the *Pseudomonas syringae* HrpA pilin, which is a subunit of a Hrp-pilus

that is 6-8 nm in diameter and is formed on bacteria in a Hrp-dependent manner (Roine

et al., "Hrp Pilus: An *hrp*-dependent Bacterial Surface Appendage Produced by

*Pseudomonas syringae* pv. *tomato* DC3000," Proc. Natl. Acad. Sci. USA 94:3459-3464

20      (1997), which is hereby incorporated by reference). The Hrp pilus is required for

pathogenicity and elicitation of the HR, and a similar structure is important for T-DNA

transfer in *Agrobacterium tumefaciens* (Fullner et al., "Pilus Assembly by

*Agrobacterium* T-DNA Transfer Genes," Science, 237:1107-1109 (1996), which is

hereby incorporated by reference). Whether these structures promote the transfer of

25      bacterial macromolecules into plant cells by serving as conduits, guides, or attachment

factors is not known.

          A current model for plant-bacterium interaction and co-evolution based

on Hrp delivery of Avr proteins into plant cells proposes that (i) Avr-like proteins are the

primary effectors of parasitism, (ii) conserved Hrp systems are capable of delivering

30      many, diverse Avr-like proteins into plant cells, and (iii) genetic changes in host

populations that reduce the parasitic benefit of an effector protein or allow its recognition

by the *R*-gene surveillance system will lead to a proliferation of complex arsenals of *avr*-

like genes in co-evolving bacteria (Alfano et al., "Bacterial Pathogens in Plants: Life Up

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Against the Wall," Plant Cell, 8:1683-1698 (1996), which is hereby incorporated by

reference). There are still many gaps in this model. For example, the physical transfer

of Avr proteins into plant cells has never been observed, the virulence functions of Avr

proteins are unknown, and it is likely that previous searches for *avr* genes in various

5      bacteria have yielded incomplete inventories of the genes in various bacteria and, thus,

incomplete inventories of the genes encoding effector proteins.

Avr proteins have not been reported outside of the cytoplasm of living

*Pseudomonas syringae* and *Xanthomonas* spp. cells (Leach et al., "Bacterial Avirulence

Genes," Annul. Rev. Phytopathol, 34:153-179 (1996); Puri et al., "Expression of

10     *avrPphB*, an Avirulence Gene from *Pseudomonas Syringae* pv. *phaseolicola*, and the

Delivery of Signals Causing the Hypersensitive Reaction in Bean," MPMI 10:247-256

(1997), which are hereby incorporated by reference), but it now appears that the Hrp

systems of *Erwinia* spp. can secrete Avr proteins in culture. A homolog of the

*Pseudomonas syringae* pv. *tomato avrE* gene has been found in *Erwinia amylovora* and

15     designated *dspA* in strain CFBP1430 and *dspE* in strain Ea321 (Gaudriault et al., "DspA,

an Essential Pathogenicity Factor of *Erwinia amylovora* Showing Homology with AvrE

of *Pseudomonas syringae*, is Secreted via the Hrp Secretion Pathway in a DspB-

dependent Way," Mol. Microbiol., 26:1057-1069 (1997); Bogdanove et al., "Homology

and Functional Similarity of a hrp-linked Pathogenicity Operon, *dspEF*, of *Erwinia*

20     *amylovora* and the *avrE* locus of *Pseudomonas syringae* Pathovar Tomato," Proc. Natl.

Acad. Sci. USA, 95:1325-1330 (1998), which are hereby incorporated by reference). *dsp*

genes are required for the pathogenicity of *Erwinia amylovora*, but not for HR

elicitation. A protein of the expected size of DspA is secreted in a Hrp- and DspB-

dependent manner by CFBP1430 (DspB is a potential chaperone) (Gaudriault et al.,

25     "DspA, an Essential Pathogenicity Factor of *Erwinia amylovora* Showing Homology

with AvrE of *Pseudomonas syringae*, is Secreted via the Hrp Secretion Pathway in a

DspB-dependent Way," Mol. Microbiol., 26:1057-1069 (1997), which is hereby

incorporated by reference). Specific antibodies were used to demonstrate

unambiguously that DspE is efficiently secreted in a Hrp-dependent manner by strain

30     Ea321 (Bogdanove et al., "*Erwinia amylovora* Secretes DspE, a Pathogenicity Factor

and Functional AvrE Homolog, Through the Hrp (Type III Secretion) Pathway," J.

Bacteriol., 180(8):2244-2247 (1998), which is hereby incorporated by reference).

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Nothing is known of the localization or expected site of action of AvrE. However, there is strong evidence that the site of action of the *Pseudomonas syringae* AvrB and AvrPto proteins is inside plant cells (see Bonas et al., "Recognition of Bacterial Avirulence Proteins Occurs Inside the Plant Cell: A General Phenomenon in Resistance

5    to Bacterial Diseases?," Plant J. 12:1 (1997); Baker et al., "Recognition and Signaling in Plant-Microbe Interactions," Science, 276:726-733 (1997), which are hereby incorporated by reference), and both proteins have now been found to be secreted by an *Erwinia chrysanthemi* Hrp system functioning heterologously in *Escherichia coli* (Ham et al., "A Cloned *Erwinia chrysanthemi* Hrp (type III Protein Secretion) System

10   Functions in *Escherichia coli* to Deliver *Pseudomonas syringae* Avr Signals to Plant Cells and to Secrete Avr Proteins in Culture," Proc. Natl. Acad. Sci. USA, 95(17): 10206-11 (1998), which is hereby incorporated by reference). This secretion is Hrp-dependent, and *Escherichia coli* cells carrying the *Erwinia chrysanthemi hrp* genes also elicit an *avrB*-dependent HR in appropriate test plants. A strong implication of this work

15   is that *Erwinia chrysanthemi*, which is a host-promiscuous soft-rot pathogen, also carries *avr*-like genes. The ability of the cloned *Erwinia chrysanthemi* Hrp system to secrete *Pseudomonas syringae* Avr proteins should promote searches for additional *avr*-like genes by providing a phenotype that is independent of plant tests, and it will enable direct investigation of Avr targeting signals and secretion mechanisms. For example,

20   chaperone-independent targeting information in two *Yersinia* Yop proteins has been shown to reside in the mRNA encoding the N-terminus of the protein (Anderson et al., "A mRNA Signal for the Type III Secretion of Yop Proteins by *Yersinia Enterocolitica*," Science, 278:1140-1143 (1997), which is hereby incorporated by reference). The involvement of similar signals in Avr secretion is suggested by the need for continued

25   protein (but not mRNA) synthesis *in planta* for Avr signal delivery, which would be consistent with a co-translational secretion process (Puri et al., "Expression of *avrPphB*, an Avirulence Gene from *Pseudomonas syringae* pv. *phaseolicola*, and the Delivery of Signals Causing the Hypersensitive Reaction in Bean," MPMI, 10:247-256 (1997), which is hereby incorporated by reference).

30          The biochemical activities or parasite-promoting functions of Avr proteins remain unclear, although several of those known make measurable contributions to virulence (Leach et al., "Bacterial Avirulence Genes," Annul. Rev. Phytopathol, 34:153-179 (1996), which are hereby incorporated by reference). Members of the

AvrBs3 family in *Xanthomonas* spp. are targeted to the plant nucleus (Van den
Ackerveken et al., "Bacterial Avirulence Proteins as Triggers of Plant Defense
Resistance," Trends Microbiol, (1997); Gabriel, "Targeting of Protein Signals from
*Xanthomonas* to the Plant Nucleus," Trends Plant Sci., 2:204-206 (1997), which are

5      hereby incorporated by reference), and some of these have been shown recently to
redundantly encode watersoaking functions associated with circulence (Yang et al.,
"Watersoaking Function(s) of XcmH1005 are Redundantly Encoded by Members of the
*Xanthomonas avr/pth* Gene Family," MPMI, 9:105-113 (1996), which is hereby
incorporated by reference. AvrD (*Pseudomonas syringae* pv. *tomato*) directs the

10     synthesis of syringolide elicitors of the HR (Leach et al., "Bacterial Avirulence Genes,"
Annul. Rev. Phytopathol, 34:153-179 (1996), which is hereby incorporated by
reference); AvrBs2 (*Xanthomonas campestris* pv. *vesicatoria*) shows similarity to *A.
tumefaciens* agrocinopine synthase (Swords et al., "Spontaneous and Induced Mutations
in a Single Open Reading Frame Alters Both Virulence and Avirulence in *Xanthomonas

15     campestris* pv. *vesicatoria avrBs2*," J. Bacteriol., 4661-4669 (1996), which is hereby
incorporated by reference); and AvrRxv (*Xanthomonas campestris* pv. *vesicatoria*) is a
homolog of AvrA (*Salmonella typhimurium*) and YopJ (*Yersinia* spp.), proteins which
travel the type III pathway in animal pathogens and trigger apoptosis in macrophages
(Hardt et al., "A Secreted *Salmonella* Protein With Homology to an Avirulence

20     Determinant of Plant Pathogenic Bacteria," Proc. Natl. Acad. Sci. USA, 94:9887-9892
(1997); Monack et al., *Yersinia* Signals Macrophages to Undergo Apoptosis and YopJ is
Necessary for this Cell Death," Proc. Natl. Acad. Sci. USA, 94:10385-10390 (1997),
which are hereby incorporated by reference. This last observation has led to the
suggestion that *avr*-R gene interactions may occur also in animal pathogenesis (Galan,

25     "'Avirulence Genes' in Animal Pathogens?," Trends Microbiol., 6:3-6 (1998), which is
hereby incorporated by reference.

        The primary sequences of the *Pseudomonas syringae* Avr proteins reveal
little about their potential function, but interestingly, when heterologously expressed in
plants, three of them have produced necrosis in test plants lacking the cognate *R* gene

30     (Gopalan et al., "Expression of the *Pseudomonas syringae* Avirulence Protein AvrB in
Plant Cells Alleviates its Dependence on the Hypersensitive Response and pathogenicity
(Hrp) Secretion System in Eliciting Genotype-specific Hypersensitive Cell Death," Plant
Cell, 8:1095-1105 (1996); Stevens et al., "Sequence Variations in Alleles of the

Avirulence Gene *avrPphE.R2* from *Pseudomonas syringae* pv. *phaseolicola* Lead to
Loss of Recognition of the AvrPphE Protein Within Bean Cells and Gain in Cultivar
Specific Virulence," Mol. Microbiol., 29(1):165-77 (1998); McNellis et al.,
"Glucocorticoid-inducible Expression of a Bacterial Avirulence Gene in Transgenic
5      *Arabidopsis* Induces Hypersensitive Cell Death," Plant J., 14(2):247-57 (1998), which
are hereby incorporated by reference). A key question is whether this results from
interaction of abnormally high levels of the bacterial protein with plant virulence targets
or with cross-reacting *R*-gene products. Further evidence suggesting that some *avr* genes
in *Pseudomonas syringae* are beneficial to the bacteria in host plants is found in recent
10     studies of *avrD* and *avrPphE*. Highly conserved, nonfunctional alleles of these genes
have been retained in pathogens whose hosts would recognize the functional Avr product
(Stevens et al., "Sequence Variations in Alleles of the Avirulence Gene *avrPphE.R2*
from *Pseudomonas syringae* pv. *phaseolicola* Lead to Loss of Recognition of the
AvrPphE Protein Within Bean Cells and Gain in Cultivar Specific Virulence," Mol.
15     Microbiol., 29(1):165-77 (1998); Keith et al., "Comparison of *avrD* Alleles from
*Pseudomonas syringae* pv. *glycinea*," MPMI, 10:416-422 (1997), which are hereby
incorporated by reference).

           Avr-like genes may function heterologously to support pathogenesis as
well as HR elicitation. The pathogenicity of an *Erwinia amylovora dspE* mutant can be
20     restored (at least partially) by a plasmid carrying the *Pseudomonas syringae avrE* locus,
suggesting that DspE and AvrE have similar functions (Bogdanove et al., "Homology
and Functional Similarity of a hrp-linked Pathogenicity Operon, *dspEF*, of *Erwinia
amylovora* and the *avrE* locus of *Pseudomonas syringae* Pathovar Tomato," Proc. Natl.
Acad. Sci. USA, 95:1325-1330 (1998), which is hereby incorporated by reference). That
25     *dspE* is essential for *Erwinia amylovora* pathogenicity, whereas *avrE* contributes only
quantitatively to the virulence of *Pseudomonas syringae* pv *tomato* (Lorang et al., "*avrA*
and *avrE* in *Pseudomonas Syringae* pv. *Tomato* PT23 Play a Role in Virulence on
Tomato Plants," MPMI, 7:508-515 (1994), which is hereby incorporated by reference),
suggests that there is less redundancy in the *Erwinia amylovora* virulence system. This
30     would be consistent with a more recent acquisition of the Hrp system by *Erwinia
amylovora* and/or a slower coevolution with its perennial hosts (Bogdanove et al.,
"Homology and Functional Similarity of a hrp-linked Pathogenicity Operon, *dspEF*, of
*Erwinia amylovora* and the *avrE* locus of *Pseudomonas syringae* Pathovar Tomato,"

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_Proc. Natl. Acad. Sci. USA_, 95:1325-1330 (1998), which is hereby incorporated by reference). The heterologous function of _Pseudomonas syringae avr_ genes in _Erwinia amylovora_ and _Erwinia chrysanthemi_ suggests that Hrp+ bacteria in the field may be able to 'sample' a buffet of _avr_-like genes from diverse sources in their coevolution with

5     changing plant populations. Many _avr_ genes have been known to be potentially mobile, because of their presence on plasmids (Vivian et al., "Avirulence Genes in Plant-Pathogenic Bacteria: Signals or Weapons?," _Microbiology_ 143:693-704 (1997); Leach et al., "Bacterial Avirulence Genes," _Annul. Rev. Phytopathol_, 34:153-179 (1996), which are hereby incorporated by reference). Recent observations with _Pseudomonas syringae_

10    highlight the apparent mobility of _avr_ genes. Several _Pseudomonas syringae avr_ genes are liked with transposable elements or phage sequences (Hanekamp et al., "Avirulence Gene D of _Pseudomonas syringae_ pv. _Tomato_ May Have Undergone Horizontal Gene Transfer," _FEBS Lett._, 415:40-44 (1997), which is hereby incorporated by reference), and the _hrp_ clusters in different strains of _Pseudomonas syringae_, although conserved in

15    themselves, are bordered by a hypervariable region enriched in _avr_ genes and mobile DNA elements.

              Alternatively, the protein or polypeptide can be a protein or polypeptide which is not normally secreted by a type III secretion system, in which case the protein or polypeptide is expressed and secreted from the host cell as a fusion protein. The

20    secretion signal, whether a mRNA or a polypeptide fragment, is linked to the protein or polypeptide of interest by an in-frame gene fusion, which preferably results in linking the mRNA or polypeptide fragment to the N-terminal end of the protein of polypeptide of interest. Such fusion proteins include a secretion signal linked to a protein or polypeptide of interest, for example, by a peptide bond between the secretion signal and

25    the protein or polypeptide of interest. Fusion proteins can be prepared by ligating two or more DNA molecules together, one of which encodes the protein or polypeptide of interest and the other of which encodes the secretion signal. The two DNA molecules must be ligated in a manner which allows their proper expression. A number of efficient expression schemes for preparing fusion proteins have been developed and are well

30    known in the art. According to one approach the fusion protein is prepared with a protease cleavage site intermediate the protein or polypeptide of interest and the secretion signal, such that the secretion signal can be removed from the protein or polypeptide of interest by, for example, proteolytic cleavage following isolation of the

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fusion protein. A linker or spacer peptide may also be included to promote proteolytic cleavage (Polyak et al., "Introduction of Spacer Peptides N-terminal to a Cleavage Recognition Motif in Recombinant Fusion Proteins Can Improve Site-specific Cleavage," Protein Eng. 10(6):615-619 (1997), which is hereby incorporated by

5      reference). A number of suitable proteases (e.g., factor Xa, chymosin, trypsin, etc.) and their protease-specific cleavage sites are known in the art, and others continually being identified. Any protease capable of cleaving a specific amino acid sequence without disrupting the protein or polypeptide of interest can be employed.

               Thus, DNA molecules encoding a protein or polypeptide capable of

10     being secreted by the type III secretion system can either be isolated (i.e., those encoding proteins that naturally contain a compatible secretion signal) or fabricated (i.e., those encoding for fusion proteins that contain a secretion signal linked by an in-frame gene fusion to a protein or polypeptide of interest). As indicated above, a single DNA construct of the present invention can contain both a type III secretion system and a

15     sequence encoding a protein or polypeptide capable of secretion by the type III secretion system or a pair of DNA constructs can be employed.

               Regardless of which approach is utilized, once the DNA molecule is constructed and obtained, it can be incorporated in cells using conventional recombinant DNA technology. Generally, this involves inserting the DNA molecule into an

20     expression system to which the DNA molecule is heterologous (i.e., not normally present). Expression systems of the present invention contain an expression vector into which is inserted one or more heterologous DNA constructs of the present invention. The heterologous DNA molecule is inserted into the expression system or vector in proper sense orientation. The vector contains the necessary elements for the

25     transcription of the DNA constructs of the present invention.

               U.S. Patent No. 4,237,224 to Cohen and Boyer, which is hereby incorporated by reference, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and

30     transfection, and replicated in cultures including prokaryotic organisms and eukaryotic cells grown in tissue culture.

Recombinant or engineered genes may also be introduced into viruses, such as vaccinia virus. Recombinant viruses can be generated by transfection of plasmids into cells infected with virus.

Suitable vectors include, but are not limited to, the following viral vectors
5    such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC184, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif, which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see Studier et. al., "Use of
10   T7 RNA Polymerase to Direct Expression of Cloned Genes," Gene Expression Technology, vol. 185 (1990), which is hereby incorporated by reference), and any derivatives thereof. Suitable vectors are continually being developed and identified. Recombinant molecules can be introduced into cells via transformation, transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the
15   vector using standard cloning procedures in the art, as described by Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, New York (1982), which is hereby incorporated by reference.

A variety of host-vector systems may be utilized to express the type III secretion system and the protein or polypeptide capable of secretion by the type III
20   secretion system. Primarily, the vector system must be compatible with the host cell used. Host-vector systems include, but are not limited to, the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g.,
25   baculovirus); and plant cells infected by bacteria or transformed via particle bombardment (i.e., biolistics). The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription elements can be used.

Different genetic signals and processing events control many levels of
30   gene expression (e.g., DNA transcription and messenger RNA (mRNA) translation).

Transcription of DNA is dependent upon the presence of a promoter which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eukaryotic promoters differ from

those of prokaryotic promoters. Furthermore, eukaryotic promoters and accompanying genetic signals may not be recognized in or may not function in a prokaryotic system, and, further, prokaryotic promoters are not recognized and do not function in eukaryotic cells.

5          Specific initiation signals are also required for efficient gene transcription and translation in prokaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector, which contains a promoter, may also contain any combination of various "strong" transcription and/or

10         translation initiation signals. Efficient translation of mRNA in prokaryotes requires a ribosome binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually ATG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and

15         probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include, but are not limited to, SD-ATG combinations synthesized by recombinant techniques, the SD-ATG combination from the cro gene or the N gene of coliphage lambda, or from the

20         *Escherichia coli* tryptophan E, D, C, B or A genes. For a review on maximizing gene expression, see Roberts and Lauer, Methods in Enzymology, 68:473 (1979), which is hereby incorporated by reference.

           Promoters vary in their "strength" (i.e. their ability to promote transcription). For the purposes of expressing a cloned DNA construct of the present

25         invention, it is desirable to use strong promoters in order to obtain a high level of transcription and, hence, expression of the DNA construct. Depending upon the host cell system utilized, any one of a number of suitable promoters may be used. For instance, when cloning in *Escherichia coli*, its bacteriophages, or plasmids, promoters such as the T7 phage promoter, lac promoter, trp promoter, recA promoter, ribosomal RNA

30         promoter, the PR and PL promoters of coliphage lambda and others, including but not limited, to lacUV5, ompF, bla, lpp, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid trp-lacUV5 (tac) promoter or other *Escherichia coli* promoters produced by recombinant DNA or other

synthetic DNA techniques may be used to provide for transcription of the inserted construct.

Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promoter unless specifically induced. In certain operons, the
5  addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the *lac* operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as trp, pro, etc., are under different controls.

Host cells can be transformed using the expression systems of the present
10  invention, whereby the host cell is transformed with one or more of the DNA constructs of the present invention, as described above. Preferably, the host cells are present in a cell culture. Although any bacterial cell is suitable for use as a host cell, *Escherichia coli*, *Erwinia amylovora*, and *Erwinia chrysanthemi* are preferred host cells.

Biological markers can be used to identify the cells carrying recombinant
15  DNA molecules. In bacteria, these are commonly drug-resistance genes. Drug resistance is used to select bacteria that have taken up cloned DNA from the much larger population of bacteria that have not. In the early mammalian gene transfer experiments involving viral genes, the transfer of exogenous DNA into cells was detected because the DNA had a biological activity, it led to production of infectious virus or produced stable
20  changes in the growth properties of the transfected cells. It was then discovered that the DNA tumor virus, herpes simplex virus (HSV), contained a gene encoding the enzyme thymidine kinase (the tk gene). The HSV tk gene can be used as a selectable genetic marker in mammalian cells in much the same way that drug-resistance genes worked in bacteria, to allow rare transfected cells to grow up out of a much larger population that
25  did not take up any DNA. The cells are transferred to selective growth medium, which permits growth only of cells that took up a functional tk gene (and the transferred DNA of interest). Various dominant selectable markers are now known in the art, including: aminoglycoside phosphotransferase (APH), using the drug G418 for selection which inhibits protein synthesis; the APH inactivates G418; dihydrofolate reductase
30  (DHFR):Mtx-resistant variant, using the drug methotrexate (Mtx) for selection which inhibits DHFR; the variant DHFR is resistant to Mtx; hygromycin-B-phosphotransferase (HPH), using the drug hygromycin-B which inhibits protein synthesis; the HPH inactivates hygromycin B; thymidine kinase (TK), using the drug aminopterin which

inhibits de novo purine and thymidylate synthesis; the TK synthesizes thymidylate;
xanthine-guanine phosphoribosyltransferase (XGPRT), using the drug mycophenolic
acid which inhibits de novo GMP synthesis; XGPRT synthesizes GMP from xanthine;
and adenosine deaminase (ADA), using the drug 9-b-D-xylofuranosyl adenine (Xyl-A)
5    which damages DNA; the ADA inactivates Xyl-A. Other selectable markers are
continually being identified.

Other aspects of the present invention relate to methods of secreting a
protein or polypeptide into the environment of a host cell. According to one
embodiment, this method is performed by introducing into a host cell a DNA construct
10   of the present invention which contains both a nucleic acid sequence encoding a
functional type III secretion system and a nucleic acid sequence encoding a protein or
polypeptide capable of being secreted by the type III secretion system. The DNA
construct is introduced into the host cell under conditions effective to cause expression of
the encoded protein or polypeptide, wherein the encoded protein or polypeptide is
15   secreted by the host cell into the environment (i.e., culture medium). According to a
second embodiment, this method is performed by introducing into a host cell a two DNA
construct system of the present invention, one of which contains a nucleic acid sequence
encoding a functional type III secretion system and the other of which contains a nucleic
acid sequence encoding a protein or polypeptide capable of being secreted by the type III
20   secretion system. The DNA constructs are introduced into the host cell under conditions
effective to cause expression of the encoded protein or polypeptide and the type III
secretion system, wherein the encoded protein or polypeptide is secreted by the host cell
into the environment.

Another aspect of the present invention relates to a method of isolating a
25   protein or polypeptide of interest. This method is performed by first providing a
recombinant host cell that contains (i) a DNA molecule encoding a functional type III
secretion system and (ii) a heterologous DNA molecule having a promoter operably
coupled to a nucleic acid sequence encoding a protein or polypeptide capable of being
secreted by the type III secretion system. The recombinant host cell is then introduced
30   into a culture medium, where the encoded protein or polypeptide and the type III
secretion system are expressed and the encoded protein or polypeptide is secreted into
the culture medium, and the encoded protein or polypeptide is isolated from the culture
medium.

The recombinant host cell can contain a homologous type III secretion system, in which case the encoded protein or polypeptide to be secreted is heterologous to both the host cell and the type III secretion system. As indicated above, the encoded protein or polypeptide can be a naturally secreted protein or a fusion protein. For

5    example, an *Erwinia amylovora* host cell (which contains a homologous hrp secretion system) can be transformed with a heterologous DNA molecule that contains a promoter operably coupled to a sequence encoding an Avr protein of *Pseudomonas syringae*. When the recombinant *Erwinia amylovora* is grown on appropriate culture medium, the Avr protein is expressed and secreted into culture medium, from which it is then isolated.

10    Alternatively, the recombinant host cell can contain a heterologous type III secretion system and a heterologous protein or polypeptide coding DNA molecule. The DNA constructs of the present invention can be used to transform a host cell. For example, an *Escherichia coli* host cell can be transformed with a single construct of the present invention which contains a DNA sequence encoding a functional type III

15    secretion system of *Erwinia chrysanthemi* and a DNA sequence encoding an Avr protein of *Pseudomonas syringae*. When the recombinant *Escherichia coli* host cell is grown on appropriate culture media, the Avr protein is expressed and secreted into culture medium, from which it is then isolated.

Effective conditions include optimal growth temperatures and nutrient

20    media which will enable maximal growth of the host cells and maximal expression of the protein or polypeptide of interest. Examplary culture media include, without limitation, LM media and minimal media, both of which are known in the art. One of ordinary skill in the art can readily determine the optimal growth temperatures for particular strains of host cells and suitable nutrient media capable of optimizing host cell growth.

25    Purified protein may be obtained by several methods. The protein or polypeptide is preferably produced in purified form (preferably at least about 80%, more preferably 90%, pure) by conventional techniques. Since the recombinant host cells express a type III secretion system, the protein or polypeptide is secreted into the growth medium of recombinant host cells. In such cases, to isolate the protein, the recombinant

30    host cells are propagated, the growth medium is centrifuged to separate cellular components from supernatant containing the secreted protein or polypeptide, and the supernatant is removed. The supernatant is then subjected to sequential ammonium sulfate precipitation. The fraction containing the polypeptide or protein is subjected to

- 30 -

gel filtration in an appropriately sized dextran or polyacrylamide column to separate the proteins. If necessary, the protein fraction may be further purified by HPLC.

Where the protein or polypeptide of interest is a fusion protein containing a protease-cleavable amino acid sequence between a secretion signal and a protein or

5    polypeptide of interest, it is possible to isolate the fusion protein, expose the isolated fusion protein to a protease, and then recover (i.e., purify) the protein or polypeptide of interest as described above. Alternatively, to avoid repetitive purification steps, it is possible to introduce a protease into the culture medium or supernatant (i.e., following separation of the cellular component from the culture medium) prior to purification.

10   After treatment with the protease, the protein or polypeptide of interest can be purified as described above.

Because the constructs and systems of the present invention enable expression of a protein or polypeptide and its secretion into an environment (e.g., culture medium) of the host cell which contains the construct or system, the present invention

15   offers significant advantages in isolating a protein or polypeptide of interest. These advantages include the ability to dispense with disruption of the host cell membrane (i.e., by sonication or other known methods) to cause release of cellular contents into the culture medium and, thus, the ability to dispense with costly purification schemes for removal of cellular debris. By avoiding these practices, significant cost savings and

20   significant improvements in protein or polypeptide purity can be recognized.

Another aspect of the present invention relates to the ability to utilize the type III secretion system in a novel manner to prospect for putative effector proteins or polypeptides. As indicated previously, Avr proteins are one type of effector protein which have significant potential for use, for example, in the field of agriculture.

25   However, prior art methods of identifying potential effector proteins have proven to be inefficient.

Accordingly, this aspect of the present invention relates to a method of identifying a gene encoding a potential effector protein or polypeptide. This method is performed by first providing a host cell that contains a DNA molecule encoding a

30   functional type III secretion system. A gene to be screened (i.e., a candidate), which encodes a protein or polypeptide, is introduced into the host cell under conditions effective to express the encoded protein or polypeptide. Next, secretion of the encoded protein or polypeptide by the recombinant host cell is determined. Secretion of the

encoded protein or polypeptide (i.e., via the type III secretion system) indicates that the gene encodes a potential effector protein or polypeptide.

The recombinant host cell can contain a homologous type III secretion system, in which case the gene to be screened is heterologous to both the host cell and

5  the type III secretion system. For example, an *Erwinia amylovora* host cell (which contains a homologous hrp secretion system) can be transformed with a heterologous gene obtained from *Pseudomonas syringae*.

Alternatively, the recombinant host cell can contain a heterologous type III secretion system and a heterologous gene to be screened. For example, an

10  *Escherichia coli* host cell can be transformed with a DNA construct that contains a DNA sequence encoding a functional type III secretion system of *Erwinia chrysanthemi* and a DNA sequence encoding a gene obtained from *Pseudomonas syringae*. (Also, co-transformation of the host cell with two separate constructs can be performed.)

A preferred approach for determining whether the protein or polypeptide

15  is expressed and secreted utilizes a chimeric gene that encodes an epitope tag fused to the protein or polypeptide. The gene to be screened can be a specific protein-coding gene or it can be obtained via shotgun cloning techniques. Regardless of how the gene is obtained, it is then modified, for example, according to the procedures of Gopalan et al., Plant Cell, 8:1095-1105 (1996), which is hereby incorporated by reference, to prepare

20  the chimeric gene. The chimeric gene is prepared in a manner which preferably results in location of the epitope tag at the C-terminal end of the fusion protein. The recombinant host cell is grown in a suitable culture medium and then all protein secreted by the recombinant host cell is isolated and, preferably, immobilized. The isolated protein is then exposed to an immunodetection assay capable of recognizing the epitope

25  tag.

Preferably the immunodetection assay utilizes a double antibody recognition complex, with the first antibody recognizing the epitope tag and the second antibody, which bears a detectable label, recognizing the first antibody. Together, the two antibodies enable detection of the epitope-tagged protein or polypeptide.

30  Monoclonal antibody production may be effected by techniques which are well-known in the art. A description of the theoretical basis and practical methodology for preparing hybridomas is set forth in Kohler and Milstein, Nature 256:495 (1975), which is hereby incorporated by reference. Procedures for raising polyclonal antibodies

are also well known, including procedures disclosed in E. Harlow, et. al., editors, Antibodies: A Laboratory Manual (1988), which is hereby incorporated by reference. In addition to utilizing whole antibodies, the processes of the present invention encompass use of binding portions of such antibodies. Such binding portions include Fab fragments,

5 F(ab')2 fragments, and Fv fragments. These antibody fragments can be made by conventional procedures, such as proteolytic fragmentation procedures, as described in Goding, Monoclonal Antibodies: Principles and Practice, pp. 98-118, New York:Academic Press (1983), which is hereby incorporated by reference.

Examples of labels useful for diagnostic imaging in accordance with the

10 present invention are radioactive labels, fluorescent labels, nuclear magnetic resonance active labels, positron emitting isotopes detectable by a positron emission tomography ("PET") scanner, chemiluminescent labels, and enzymatic markers. A number of labels are well known in the art and others are continually being identified.

This method of screening for putative effector proteins enables one of

15 ordinary skill in the art to more readily identify putative effector proteins, which can then be tested on host organisms to determine the ability of the putative effector protein to induce a host plant response.

These aspects of the present invention are further illustrated by the examples below.

20

## EXAMPLES

The following examples are provided to illustrate embodiments of the present invention but they are by no means intended to limit its scope.

25 **Materials and Methods**

**Bacterial strains, culture conditions, and DNA manipulation techniques:**

Bacterial strains and plasmids used in this study are listed in Table 1 below.

Table 1: Bacterial Strains and Plasmids

| Designation | Relevant Characteristics and Use | Reference or Source |
|---|---|---|
| *Escherichia coli* | | |
| DH5α | SupE44 ΔlacU169 (f80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 | Life Technologies |

| Designation | Relevant Characteristics and Use | Reference or Source |
|---|---|---|
| | relA1, Nx$^r$ | |
| XLOLR | Δ(mcrA)183 Δ(mcrBc-hsdSMR-mrr)173 endA1 thi-1 recA1 gyrA96 relA1 lac [F' proAB laqI$^q$ZΔM15 Tn10(Tc$^r$)] | Stratagene |
| SM10 λpir | SM10 lysogenized with λ-pir for mobilizing pUT::mini-Tn5Cm, Km$^r$ | (Miller et al., J. Bacteriol., 170: 2575-2583 (1988), which is hereby incorporated by reference) |
| MC4100 | F' araD139 Δ(argF-lacZYA)U169 rpsL 150 relA1 fib-5301 ptsF25 deoC1 | (Casadaban, J. Mol. Biol. 104:541-555 (1976), which is hereby incorporated by reference) |
| **Plasmids** | | |
| pFLAG-CTC | For construction of C-terminal fusion to FLAG peptide, Ap$^r$ | Kodak Scientific Imaging Systems |
| pUT::mini-Tn5Cm | Mini-Tn5 transposon with Cm$^r$ on suicide plasmid pGP704 derivative for transposon mutagenesis, Ap$^r$ | (de Lorenzo et al., J. Bacteriol., 172:6568-6572 (1990), which is hereby incorporated by reference) |
| pML123 | Broad host range expression vector for cloning avrPto-FLAG, Gm$^r$ | (Labes et al., Gene, 89:37-46 (1990), which is hereby incorporated by reference) |
| pPtE6 | An avrPto clone in pDSK519, Km$^r$ | (Ronald et al., J. Bacteriol. 174: 1604-1611 (1992), which is hereby incorporated by reference) |
| pHIR11 | pLAFR3 carrying Pss61 hrp/hrc cluster, Tc$^r$ | (Huang et al., J. Bacteriol., 170: 4748-4756 (1988), which is hereby incorporated by reference) |
| pCPP2156 | pCPP19 carrying E. chrysanthemi hrp cluster, Sp$^r$ | This work |
| pCPP2157 | pCPP19 carrying E. chrysanthemi hrp cluster, Sp$^r$ | (Bauer et al., MPMI 8:484-491 (1995), which is hereby incorporated by reference) |
| pCPP2329 | pFLAG-CTC carrying avrPto, Ap$^r$ | This work |
| pAVRB-FLAG2 | pML123 carrying avrB-FLAG, Gm$^r$ | (Gopalan et al., Plant Cell, 8:1095-1105 (1996), which is hereby incorporated by reference) |
| pAVRB1 | pDSK519 carrying avrB, Km$^r$ | (Tamaki et al., J. Bacteriol. 170:4846-4854 (1988), which is hereby incorporated by reference) N.T. Keen |
| pAVRPTO-FLAG | pML123 carrying avrPto-FLAG, Gm$^r$ | This work |
| pCPP2368 | A pCPP2156::Tn5Cm that has HR$^-$ | This work |

| Designation | Relevant Characteristics and Use | Reference or Source |
|---|---|---|
| | phenotype, Sp$^r$, CM$^r$ | |
| pCPP2416 | A pCPP2156::Tn5Cm that has HR$^+$ phenotype, Sp$^r$, Cm$^r$ | This work |
| pCPP2318 | pCPP30 carrying mature blaM, Tc$^r$ | (Charkowski et al., J. Bacteriol. 179:3866-3874 (1997), which is hereby incorporated by reference) |

*Escherichia coli* strains were routinely grown in LM medium (Hanahan, D., J. Mol.
Biol., 166:557-580 (1983), which is hereby incorporated by reference) at 37°C for
isolation of plasmids and at 30°C for protein secretion assays. The following

5    concentrations of antibiotics were used in selective media: ampicillin (Ap), 100 µg/ml;
chloramphenicol (Cm), 20 µg/ml; gentamycin (Gm), 10 µg/ml; kanamycin (Km), 50
µg/ml; nalidixic acid (Nx), 20 µg/ml; spectinomycin (Sp), 50 µg/ml; tetracycline (Tc), 10
µg/ml. Standard procedures were followed by DNA manipulations (Sambrook, J., et al.,
Molecular Cloning: A Laboratory Manual, 2$^{nd}$ Edition (Cold Spring Harbor Laboratory

10   Press, Cold Spring Harbor, NY), pp Pages (1989), which is hereby incorporated by
reference).

**Mini-Tn5Cm mutagenesis of the hrp gene cluster in pCPP2156:**

Mini-Tn5Cm mutagenesis of *Escherichia coli* DH5α(pCPP2156) was
initiated by conjugation with *Escherichia coli* SM10λpir (pUT::mini-Tn5Cm). Because

15   pUT cannot replicate in *Escherichia coli* DH5α, Cm$^r$ transconjugants have mini-Tn5Cm
transposed to the chromosome or pCPP2156. To obtain a pool of pCPP2156::mini-
Tn5Cm plasmids, all Cm$^r$ colonies were triparentally mated with *Escherichia coli*
XLOLR (Tc$^r$). The cosmids from *Escherichia coli* XLOLR transconjugants, selected on
LM agar containing Tc, Sp, and Cm, were isolated and their restriction fragment patterns

20   compared with each other and pCPP2156. All 46 cosmids initially examined contained
random insertions of mini-Tn5Cm in pCPP2156. Two primers were used to sequence
from both ends of mini-Tn5Cm including a first primer having a nucleotide sequence
corresponding to SEQ. ID. No. 3 as follows:

AGATCTGATC AAGAGACAG                                              19

25   and a second primer having a nucleotide sequence corresponding to SEQ. ID. No. 4 as
follows:

CCGTGTGTAT AAGAGTCAG                                              19

Based on restriction mapping and DNA sequencing from both ends of mini-Tn5Cm, two

different pCPP2156:mini-Tn5Cm derivatives were chosen. In one of them, Tn5Cm was

inserted in the intergenic region between hrpJ and hrcV in the hrpJ operon. This cosmid

was named pCPP2368. The other cosmid contained mini-Tn5Cm outside of the hrp

5    cluster and was named pCPP2416. Both cosmids were electroporated into Escherichia

coli DH5α.

**Plant bioassays:**

Tobacco (Nicotiana tabacum L. cv. Xanthi) and Nicotiania clevelandii

were grown under greenhouse conditions and then maintained in the lab at room

10   temperature with daylight and supplemental metal halide illumination for HR assays.

Soybean (Glycine max L.) and tomato (Lycopersicum esculentum Mill. cv. Rio Grande)

plants were grown from seeds in pots with Cornell Mix (Cornell University) in the lab at

room temperature. Escherichia coli DH5α cells grown overnight on LM plates were

washed twice with 5mM MES (morpholinoethanesulfonic acid, pH 6.5) by

15   centrifugation and then resuspended in an appropriate volume of the same buffer to an

$OD_{600}$ of 0.8 (experiments involving avrPto used Escherichia coli MC4100 and a 3-fold

higher level of inoculum). Previously described procedures were used for the infiltration

of bacterial cells into tobacco, tomato and Nicotiana clevelandii leaves (Bauer et al.,

MPMI, 7:573-581 (1994), which is hereby incorporated by reference) and soybean

20   leaves (Gopalan et al., Plant Cell, 8:1095-1105 (1996), which is hereby incorporated by

reference).

**Preparation of AvrB antibodies:**

AvrB-FLAG was purified from Escherichia coli DH5α(pFLAG-

CTC::AvrB) by affinity chromatography as described (Gopalan et al., Plant Cell, 8:1095-

25   1105 (1996), which is hereby incorporated by reference), followed by precipitation of

aliquots containing 1 mg of partially purified protein with trichloroacetic acid ("TCA")

(20% final concentration), resuspension in SDS polyacrylamide gel loading buffer

(Sambrook, J., et al., Molecular Cloning: A Laboratory Manual, 2nd Edition (Cold

Spring Harbor Laboratory Press, Cold Spring Harbor, NY), (1989), which is hereby

30   incorporated by reference), and electrophoresis on 1.5 mm X 11 cm X 10 cm 12%

polyacrylamide preparative gels. The AvrB band was excised from each gel following

brief staining with a solution of 0.2% Coomassie R350 (Pharmacia Biotech) dissolved in

water. Subsequent extraction of AvrB from the gel matrix and generation of polyclonal

rabbit anti-AvrB antisera were performed by the Immunological Resource Center at the University of Illinois, Urbana IL. Prior to usage, the antisera was delipified with sodium dextran sulfate (average molecular weight of 500,000) to a final concentration of 0.25% and CaCl$_2$ to a final concentration of 1.0% followed by incubation of 4°C for 8-12 hours

5    (Walton, K.W., et al., J. Clin. Pathol., 17:627-643 (1964), which is hereby incorporated by reference). This mixture was clarified by centrifugation at 12,000 X g at 4°C for 10 minutes. Proteins were precipitated by the addition of ammonium sulfate at 50% saturation, followed by incubation at 4°C for 8-12 hours, and then collected by centrifugation at 12,000 X g at 4°C for 10 minutes and resuspended in their original

10   volume with phosphate-buffered saline.

**Construction of pAVRPTO-FLAG:**

The *avrPto* gene was isolated by polymerase chain reaction ("PCR") with *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and pPtE6 as the template. The upper primer had a nucleotide sequence that contains an *Nde* I site, corresponding to SEQ. ID.

15   No. 5 as follows:

GAGCGAGCAT ATGGGAAATA TATGTGTCGG C                                        31

The lower primer had a nucleotide sequence that contains an *Sal* I site, corresponding to SEQ. ID. No. 6 as follows:

ATTGTAGTCG ACTTGCCAGT TACGGTACGG G                                        31

20   The reaction products from 30 PCR cycles were resolved by electrophoresis through 0.7% agarose, and the *avrPto* DNA was isolated using an Eluquick kit (Schleicher & Schuell), followed by digestion with *Nde* I and *Sal* I. This DNA was cloned into pFLAG-CTC, previously digested with *Nde* I and *Sal* I, and named pCPP2329. The *avrPto-FLAG* DNA was isolated from pCPP2329 by digestion with *Ssp* I and cloned into

25   pML 123, which had been previously digested with *BamH* I and blunted with Klenow polymerase, producing pAVRPTO-FLAG. As with *avrB-FlaG2* (Gopalan, S., et al., Plant Cell, 8:1095-1105 (1996), which is hereby incorporated by reference), *avrPto-FLAG* is expressed by both the tax promoter (from pFLAG-CTC) and the pML 123 *nptII* promoter, thus permitting constitutive expression in LM medium and *in planta*.

30   **Preparation of protein samples from supernatant and cell fractions:**

Bacteria grown overnight on LM plates at 37°C were washed twice by centrifugation and resuspended in LM broth. Each bacterial suspension was diluted to

$OD_{600}$ = 0.2 in 40 ml of LM broth containing appropriate antibiotics and cultured at 30°C in a rotary shaking incubator at 220 rpm until the $OD_{600}$ reached 0.8. Centrifugations for the separation of bacterial cultures into cell-bound and supernatant fractions were performed with an SS-34 rotor (DuPont Instrument) at 4°C.

5        Forty milliliters of culture was initially centrifuged at 6,000 rpm (> 4300 x g) for 15 minutes. For the supernatant fraction, the upper 20 ml of supernatant was carefully transferred to a new centrifuge tube and further centrifuged at 12,000 rpm (> 17200 x g) for 40 minutes, followed by transfer of the upper 10 ml of supernatant to a new tube. Six milliliters of 25% TCA was added to the supernatant fraction, which was

10      then kept on ice for 3-4 hours, followed by centrifugation at 12,000 (> 17200 x g) rpm for 40 minutes. The pellet was subsequently washed with 20 ml of ice-cold acetone and then resuspended in 200 µl or 100 µl X SDS sample buffer (New England Biolabs). For the cell fraction, the pellet from the initial centrifugation was resuspended in 4 ml of LM broth. One hundred microliters of bacterial cell suspension was mixed with 50 µl of 3 X

15      SDS sample buffer. Each protein sample was held in a boiling water bath for 5 minutes before electrophoresis, and then 15 µl of each sample was loaded onto the gel.

**Immunoblot analysis:**

Protein samples were separated by electrophoresis through a 10% SDS-polyacrylamide gel. Proteins in the gel were then electrotransferred to Immobilon-P

20      membrane (Millipore Corp.) with a Semi-Phor system (Hoefer Scientific Instruments). AvrB-FLAG and AvrPto-FLAG were detected with the Western-Light Plus kit (Tropix) using anti-FLAG M2 antibodies (Kodak Scientific Imaging Systems) and anti-mouse IgG alkaline phosphatase conjugate (Sigma) as primary and secondary antibodies, respectively. AvrB and β-lactamase were detected with the same system except using

25      anti-AvrB antibodies or anti-β-lactamase antibodies (5 prime → 3 prime) and anti-rabbit IgG alkaline phosphatase conjugate (Sigma) as primary and secondary antibodies, respectively.

**Primers, DNA sequencing and data analysis:**

Oligonucleotide synthesis and DNA sequencing were performed at the

30      Cornell Biotechnology Center. DNA sequence data were managed and analyzed with the DNAStar Program (DNAStar, Madison, WI).

<u>Example 1</u> -    Preparation of Cosmid pCPP2156, Which Carries an Intact *Erwinia chrysanthemi hrp* Gene Cluster

Several cosmids carrying *Erwinia chrysanthemi* EC16 *hrp* genes were previously isolated on the basis of their ability to hybridize with an *Erwinia amylovora*

5      DNA fragment carrying the *hrpJ* operon (Bauer, D.W., et al., <u>MPMI</u>, 7:573-581 (1994), which is hereby incorporated by reference). One of these cosmids, pCPP2157, is shown in Figure 1 and was subsequently found to carry also *hrpN* and the complete *hrpC* operon (Bauer, et al., "*Erwinia chrysanthemi* harpin$_{Ech}$: An Elicitor of the Hypersensitive Response that Contributes to Soft-rot Pathogenesis," 8:484-491 (1995);

10     Kim, et al., "The hrpC and HrpN Operons of *Erwinia chrysanthemi* EC16 are Flanked by picA and Homologs of Hemolysin/Adhesion Genes and Accompanying Activator/Transporter Genes," <u>MPMI</u>, 11(6):563-567 (1998), which are hereby incorporated by reference). Although pCPP2157 appeared to carry both borders of the *Erwinia chrysanthemi hrp* cluster, *Escherichia coli*(pCPP2157) failed to elicit an HR in

15     tobacco leaves. DNA sequencing of the right end of the pCPP2157 insert revealed that *hrcU* was missing the last 180 nucleotides, as suggested by comparison with the *Erwinia amylovora hrcU* gene (Bogdanove, et al., "*Erwinia amylovora* Secretes Harpin via a Type III Pathway and Contains a Homolog of YopN of Yersinia spp.," <u>J. Bacteriol.</u>, 178:1720-1730 (1996), which is hereby incorporated by reference). *hrcU* is one of nine

20     former *hrp* genes that encode core components of the type III secretion system, are broadly conserved in plant and animal pathogenic bacteria, and have been renamed as *hrc* (HR and conserved) genes (Bogdanove, et al., <u>Mol. Microbiol.</u>, 20:681-683 (1996), which is hereby incorporated by reference). Because of the *hrcU* truncation, additional cosmids hybridizing with probes carrying *hrpN* and *hrcU* were analyzed. pCPP2156 was

25     one of those. Partial DNA sequence analysis and physical map comparisons with the *Erwinia amylovora hrp* genes suggested that pCPP2156 carried the entire *Erwinia chrysanthemi hrp* gene cluster, including at least one intercalated region not obviously related to Hrp function and 14-kb of additional DNA beyond *hrcU* (Figure 1). However, pCPP2156 failed to elicit an HR in tobacco.

30

**Example 2 -** *Escherichia coli*(pCPP2156) **Enables Elicitation of an AvrB-
dependent HR in *Nicotiana clevelandii* and Soybean Cultivar
Norchief**

The plasmid pHIR11, which carries the intact *Pseudomonas syringae* pv

5     *syringae* 61 *hrp* cluster, enables *Escherichia coli* to elicit an HR in tobacco, because it

also carries *hrmA*, an *avr*-like gene whose transient expression in tobacco cells is lethal

(Alfano, et al., Mol. Microbiol., 19:715-728 (1996); Alfano, et al., MPMI, 10:580-588

(1997), which are hereby incorporated by reference). This suggested the possibility that

pCPP2156 failed to elicit an HR in tobacco because it did not carry an appropriate *avr*

10     gene. To test this, pAVRB-FLAG2 was transformed into *Escherichia coli* DH5α cells

carrying either pCPP2156 or pCPP2157. pAVRB-FLAG2 expresses the *Pseudomonas*

*syringae* pv *glycinea avrB* gene such that the product has an eight-amino acid FLAG

epitope C-terminal fusion (Gopalan, et al., Plant Cell, 8:1095-1105 (1996), which is

hereby incorporated by reference). Transformants were infiltrated at a concentration of 5

15     X $10^8$ cfu/ml into *Nicotiana clevelandii*, a plant that reacts hypersensitively to Hrp$^+$

bacteria carrying *avrB*. A typical HR developed within 24 hours in panels inoculated

with bacteria carrying both *avrB* and pCPP2156, but there was no response in panels

inoculated with bacteria lacking *avrB* or carrying an incomplete *hrp* cluster (Figure 2A),

or inoculated with bacteria carrying only pAVRB-FLAG2.

20     The failure of *Escherichia coli*(pCPP2157, pAVRB-FLAG2) to elicit an

HR in *Nicotiana clevelandii* suggested that this ability was Hrp-dependent. However, an

explanation based on differences in the DNA flanking the *hrp* gene clusters in pCPP2156

and pCPP2157 remained a formal possibility. To resolve this, pCPP2156 was mutated

with mini-Tn5*Cm* and two derivatives were isolated. Restriction mapping and DNA

25     sequence analysis revealed that pCPP2416 and pCPP2368 carried insertions in the 14-kb

region beyond *hrcU* and in the intergenic region between *hrpJ* and *hrcV*, respectively

(Figure 1). The mutation in pCPP2368 would be expected to block transcription of *hrcV*

and downstream genes in the putative *hrpJ* operon, and a polar mutation in the *hrpJ*

operon of *Pseudomonas syringae* pv *syringae* 61 has been shown to result in

30     accumulation of the HrpZ harpin within the bacterial cytoplasm (Charkowski et al.,

"Altered Localization of HrpZ in *Pseudomonas syringae* pv. *syringae hrp* Mutants

Suggests That Different Components of the Type III Secretion Pathway Control Protein

Translocation Across the Inner and Outer Membranes of Gram-negative Bacteria," J.

Bacteriol., 179:3866-3874 (1997), which is hereby incorporated by reference).

*Escherichia coli* cells carrying pCPP2416 and pCPP2368 were transformed with
pAVRB-FLAG2 and tested for their ability to elicit the HR in *Nicotiana clevelandii*. An
HR was observed only with *Escherichia coli* (pCPP2416, pAVRB-FLAG2) (Figure 2A).

To further test the ability of *Escherichia coli* (pCPP2156) to elicit an

5    *avrB*-dependent HR, the leaves of soybean cultivars Norchief (*RPG1*) and Acme (*rpg1*)
were infiltrated with bacteria carrying various plasmids. *RPG1* is an *R* gene that
interacts in a gene-for-gene manner with *avrB* (Staskawicz, et al., J. Bacteriol.,
169:5789-5794 (1987), which is hereby incorporated by reference). The HR was
observed only in Norchief inoculated with bacteria carrying both *avrB* and an intact *hrp*

10   cluster (Figure 2B).


**Example 3 -   *Escherichia coli*(pCPP2156) Secretes AvrB in Culture in a Hrp-
dependent Manner while Retaining β-lactamase**

The secretion of Avr proteins by *Pseudomonas syringae* is presumed to be

15   dependent on host cell contact, because Avr proteins remain cytoplasmic in culture even
when the Hrp system is actively secreting harpins (Alfano, et al., J. Bacteriol., 179:5655-
5662 (1997), which is hereby incorporated by reference). The seemingly less specialized
interaction of *Erwinia chrysanthemi* with its hosts suggested the possibility that Avr
secretion may be regulated less tightly. To test this, FLAG epitope-tagged AvrB

20   encoded by pAVRB-FLAG2 was used to determine if *Escherichia coli* (pCPP2156)
could secrete AvrB in culture. Bacterial cultures in late logarithmic-phase growth were
separated into supernatant and cell-bound fractions by centrifugation, and then proteins
in both fractions were resolved by SDS-PAGE. AvrB-FLAG was visualized by
immunoblotting with anti-FLAG monoclonal antibodies and chemiluminescent

25   detection. AvrB-FLAG was found in the supernatant of *Escherichia coli*(pCPP2156)
(Figure 3). Although much of the AvrB remained in the cell-bound fraction, secretion
was Hrp-dependent and specific in that no AvrB-FLAG was found in the supernatant of
*Escherichia coli* (pCPP2368) (Figure 3), and Coomassie staining revealed equally low
levels of protein in the supernatant fractions of all of the bacteria tested.

30           To confirm that the presence of AvrB-FLAG in the *Escherichia
coli*(pCPP2156) medium resulted from specific secretion and not cell lysis, and that
secretion was not due to FLAG epitope, the localization of AvrB and mature β-lactamase
was simultaneously monitored (Figure 4). *Escherichia coli* cells carrying pCPP2156 or
pCPP2368 were first transformed with pAVRb1, which expresses AvrB from the *lac*

promoter (Tamaki, S., et al., J. Bacteriol., 170:4846-4854 (1988), which is hereby

incorporated by reference), and pCPP2318, which encodes a mature β-lactamase that

lacks its N-terminal signal peptide and can be used as a cytoplasmic marker (Charkowski

et al., "Altered Localization of HrpZ in *Pseudomonas syringae* pv. *syringae hrp* Mutants

5        Suggests That Different Components of the Type III Secretion Pathway Control Protein

Translocation Across the Inner and Outer Membranes of Gram-negative Bacteria," J.

Bacteriol., 179:3866-3874 (1997), which is hereby incorporated by reference). The

distribution of AvrB and β-lactamase in the same supernatant and cell-bound fraction

samples was monitored by immunoblotting with appropriate antibodies. The

10       *Escherichia coli*(pCPP2156) supernatant sample contained AvrB but no β-lactamase

(Figure 4), indicating that AvrB secretion occurred without the FLAG epitope and

without cell lysis.


15  **Example 4 -** *Escherichia coli*(pCPP2156) Secretes AvrPto in Culture in a Hrp-
            dependent Manner

            The evidence for Avr action inside plant cells following Hrp-dependent

transfer is strongest with AvrPto, whose structural gene was originally isolated from

*Pseudomonas syringae* pv. *tomato* (Alfano, et al., J. Bacteriol., 179:5655-5662 (1997);

Tang, et al., Science, 274:2060-2062 (1996); Scofield, et al., Science, 274:2063-2065

20       (1996); Ronald, et al., J. Bacteriol., 174:1604-1611 (1992), which are hereby

incorporated by reference). Consequently, AvrPto was selected to test whether the

ability of *Escherichia coli*(pCPP2156) to deliver *Pseudomonas syringae* Avr signals *in

planta* and to secrete Avr proteins in culture would extend beyond AvrB. First, the

construct pAVRPTO-FLAG, which encodes AvrPto with a C-terminal FLAG epitope

25       fusion, was prepared. *Escherichia coli* cells carrying pAVRPTO-FLAG and pCPP2156

(but not pAVRPTO-FLAG alone, pCPP2156 alone, or pAVRPTO-FLAG with

pCPP2368) elicited an HR in tomato cultivar Rio Grande carrying the *Pto* resistance

gene. The secretion of AvrPto was determined with the same methods used for AvrB-

FLAG and AvrB. AvrPto-FLAG was secreted by *Escherichia coli*(pCPP2156) but not

30       by *Escherichia coli*(pCPP2157) or *Escherichia coli*(pCPP2368) (Figure 5). Thus,

secretion of AvrPto-FLAG was Hrp-dependent, and it also occurred without leakage of

β-lactamase. In contrast, no AvrPto-FLAG was found in the supernatant of *Escherichia

coli*(pHIR11), which expresses the intact *Pseudomonas syringae* Hrp system (Figure 5).

Th  isolation of a cluster of *Erwinia chrysanthemi hrp* genes that directs *Escherichia coli* to secrete *Pseudomonas syringae* Avr proteins in culture and deliver Avr signals in planta has several implications for the pathogenic biology of *Erwinia chrysanthemi* and *Pseudomonas syringae*. As representative necrotrophic and biotrophic

5    parasites, respectively, these two bacteria mark the extremes in the pathogenic personalities of the common gram-negative phytopathogenic bacteria. Nevertheless, it is possible that they elicit the HR and initiate parasitic attack in fundamentally similar ways, that they may be able to interchange their *avr* genes without loss of function, and that their cloned *hrp* clusters can be used to biochemically investigate Avr protein

10   secretion and to systematically prospect for the proteins injected into plants by many plant pathogenic bacteria.

*Erwinia chrysanthemi* and *Pseudomonas syringae* appear to elicit the HR by the same mechanism in that their cloned *hrp* clusters are dependent on an appropriate *avr* gene for elicitation of the HR when heterologously expressed in nonpathogens. Thus,

15   cosmid pHIR11 (*Pseudomonas syringae hrp* cluster) directs HR elicitation in tobacco, because it carries the *avr*-like *hrmA* gene. Cosmid pCPP2156 (*Erwinia chrysanthemi hrp* cluster) fails to elicit the Hr in tobacco, *Nicotiana clevelandii*, soybean, or tomato because it does not carry an *avr* gene that is recognized by these plants, but when provided with *avrB* or *avrPto* it appropriately directs elicitation of the HR in *Nicotiana*

20   *clevelandii*, soybean cultivar Norchief, and tomato cultivar Rio Grande. This has two implications regarding HR elicitation by *Erwinia chrysanthemi*. First, the harpin encoded by pCPP2156, like that encoded by pHIR11, is apparently insufficient for bacterial HR elicitation (although both harpins can elicit programmed cell death when delivered exogenously) (Bauer, et al., "*Erwinia chrysanthemi* harpin$_{Ech}$: An Elicitor of

25   the Hypersensitive Response that Contributes to Soft-rot Pathogenesis," 8:484-491 (1995); He, et al., Cell, 73:1255-1266 (1993), which are hereby incorporated by reference). Second, *Erwinia chrysanthemi* must carry *avr* genes somewhere outside the region cloned in pCPP2156 because it is able to elicit a Hrp-dependent HR in tobacco without provision of a heterologous *avr* gene (Bauer, et al., MPMI, 7:573-581 (1994),

30   which is hereby incorporated by reference).

*Erwinia chrysanthemi hrp* mutants also are reduced in their ability to elicit infection at low levels of inoculum. Because it now appears that the primary function of the Hrp system is to deliver Avr-like proteins to host cells, identifying these proteins and

determining their function will be key to understanding how *Erwinia chrysanthemi* initiates infection. Recent observations with *Erwinia amylovora* indicate that homologous *avr*-like genes are present in *Erwinia* spp. and *Pseudomonas syringae* (Gaudriault, et al., Mol. Microbiol., 26:1057-1069 (1997); Bogdanove, et al., Proc. Natl.

5    Acad. Sci. USA, 95:1325-1330 (1998), which are hereby incorporated by reference). Specifically, *dspE*, which is required for the pathogenicity of *Erwinia amylovora*, is a homolog of *avrE*, a gene that contributes quantitatively to the virulence of *Pseudomonas syringae* pv *tomato* strain PT23 on tomato and has an Avr phenotype in *Pseudomonas syringae* pv *glycinea* when tested on a variety of soybean cultivars (Lorang, et al.,

10   MPMI, 7:508-515 (1994); Lorang, et al., MPMI, 8:49-57 (1995), which are hereby incorporated by reference). The ability of *avrE* to restore the pathogenicity of an *Erwinia amylovora dspE* mutant provides direct evidence that a *Pseudomonas syringae avr* gene can function biologically in an *Erwinia* background. (Bogdanove, et al., Proc. Natl. Acad. Sci. USA, 95:1325-1330 (1998), which is hereby incorporated by reference).

15   Furthermore, DspE-specific antibodies and appropriate *hrp* mutants have been used to establish that *Erwinia amylovora* secretes DspE in a Hrp-dependent manner in culture (Bogdanove, et al., J. Bacteriol. 180:2244-2247 (1998), which is hereby incorporated by reference). however, it is not known whether AvrE can be secreted in culture by *Erwinia amylovora* (or *Pseudomonas syringae*) or whether DspE and AvrE function inside plant

20   cells.

The regulation of the *Erwinia chrysanthemi* Hrp system appears more relaxed in two ways in comparison with host-specific pathogens like *Pseudomonas syringae*. First, the *Erwinia chrysanthemi hrp* genes are not repressed by complex media (which enhances the utility of the system for secretion studies) (Collmer, et al., in "Advances in

25   Molecular Genetics of Plant-Microbe Interactions," Vol. 3 ed. Daniels, M.J. (Kluwer, Dordrecht), pp. 49-56 (1994), which is hereby incorporated by reference). Second, the *Erwinia chrysanthemi* Hrp system does not appear to be gated in culture with respect to the secretion of Avr proteins. Although the *Pseudomonas syringae hrp* cluster carried on pHIR11 enables delivery of AvrB and AvrPto signals (presumably the Avr proteins

30   themselves) to plant cells, it does not direct secretion of these proteins in culture (Figure 5) (Gopalan, et al., Plant Cell, 8:1095-1105 (1996), which is hereby incorporated by reference). Because *Erwinia chrysanthemi* and *Pseudomonas syringae* possess similar Hrp systems (both in group I) (Alfano, et al., J. Bacteriol., 179:5655-5662 (1997);

Kim, et al., "The hrpC and hrpN Operons of *Erwinia chrysanthemi* EC16 are Flanked by

plcA and Homologs of Hemolysin/Adhesion Genes and Accompanying

Activator/Transporter Genes," MPMI 11(6):563-567 (1998), which are hereby

incorporated by reference), comparisons and genetic exchanges between them are likely

5      to be useful for elucidating the mechanisms controlling Avr protein secretion in

*Pseudomonas syringae*.

        There is yet no direct evidence for the Hrp-mediated transfer of any Avr protein

into plant cells, although the indirect evidence for this is particularly compelling with

AvrB and AvrPto, as discussed above. The observation that these two proteins can travel

10     the Hrp pathway to the bacterial milieu now provides direct confirmation of the first step

in the translocation process. More importantly, the targeting signals controlling secretion

and other aspects of the secretion process now can be explored *in vitro*. In this regard,

the differing traffic specificities of the type II and type III protein secretion systems of

*Erwinia chrysanthemi* are noteworthy, especially since both systems function

15     heterologously in *Escherichia coli*. The cloned cluster of out (type II secretion) genes

from *Erwinia chrysanthemi* EC16 directs the secretion of pectate lyase isozymes

expressed from *Erwinia chrysanthemi pel* genes but not from *Erwinia carotovora pel*

genes (He, et al., Proc. Natl. Acad. Sci. USA, 88:1079-1083 (1991), which is hereby

incorporated by reference). This species-specific secretion occurs despite the fact that

20     the Out systems and some of the Pels of these two species are homologous (Lindeberg, et

al., Mol. Microbiol., 20:175-190 (1996), which is hereby incorporated by reference).

The construction of hybrid Pels has shown that the targeting information controlling

species-specific secretion resides in the tertiary structure of these proteins (Lindeberg, et

al., J. Bacteriol. 180:1431-1437 (1998), which is hereby incorporated by reference). In

25     contrast, the *Erwinia chrysanthemi* Hrp (type III) system lacks even genus-specificity for

its traffic, and the secreted proteins may be devoid of targeting information. This is

based on the possibility that targeting information resides in the mRNA encoding the N-

termini of these proteins, as has been demonstrated recently for the YopE and YopN

proteins secreted by the *Yersinia* type III pathway (Anderson, et al., Science, 278:1140-

30     1143 (1997), which is hereby incorporated by reference). Use of the cloned *Erwinia*

*chrysanthemi* Hrp secretion system should make testing this hypothesis and the

identification of targeting signals straightforward.

*Escherichia coli* heterologously expressing the *Erwinia chrysanthemi* Hrp system also can be used to systematically prospect for genes from *Erwinia chrysanthemi*, *Pseudomonas syringae*, and possibly other bacteria that encode Avr-like effector proteins. However, two factors may limit universal application of this system. First,

5   some Avr-like proteins may require a dedicated chaperone, as has been observed with *Yersinia* Yops (although characteristic gene arrangements and structural properties of the chaperones may help identify them) (Wattiau, et al., Mol. Microbiol., 20:255-262 (1996), which is hereby incorporated by reference). Second, it is not known whether *Escherichia coli*(pCPP2156) will secrete Avr-like proteins derived from pathogens like

10  *Ralstonia solanacearum* and *Xanthomonas* spp., which posses group II Hrp systems (Alfano, et al., J. Bacteriol., 179:5655-5662 (1997), which is hereby incorporated by reference).

**Example 5 -**   **Secretion of DspE by the *Erwinia amylovora* Hrp System**

15          The *Erwinia amylovora* DspE (DspA in Gaudriault et al., Mol. Microbiol. 26:1057-1069 (1997), which is hereby incorporate by reference) protein is required for pathogenicity and has homology to AvrE of *Pseudomonas syringae* pv. tomato (Bogdanove et al., Proc. Natl. Acad. Sci. USA 95:1325-1330 (1998); Gaudriault et al., Mol. Microbiol. 26:1057-1069 (1997), which are hereby incorporate by reference). In

20  *Pseudomonas syringae* pv. *glycinia*, DspE acts as an avirulence gene, converting it to avirulence on its host, soybean. In addition, avrE restores pathogenicity to an *Erwinia amylovora* dspE mutant (Bogdanove et al., Proc. Natl. Acad. Sci. USA 95:1325-1330 (1998), which is hereby incorporated by reference). Thus, DspE acts as a virulence protein in host plants and as an avirulence protein in a nonhost plant. To determine

25  whether DspE was secreted by the *Erwinia amylovora* Hrp system, strains Ea273 (the wild-type strain), Ea273-K178 (a hrp secretion mutant), and Ea273dspEÆ1521 (containing a 1,521 bp in-frame deletion in the 3' portion of DspE (Bogdanove et al., J. Bacteriol. 180:2244-2247 (1998); Bogdanove et al., Proc. Natl. Acad. Sci. USA 95:1325-1330 (1998), which are hereby incorporated by reference) were grown in hrp gene-

30  inducing minimal medium. Analysis of the proteins from the cell and supernatant fractions on immunoblots revealed that DspE and DspEÆ1521 were present in the culture supernatant of Ea273 and Ea273dspEÆ1521, respectively (Figure 6). In contrast, no DspE was detected in the supernatant of the hrp mutant (Ea273-K178), but it was

detected within the bacterial cells. Therefore, the DspE protein is secreted in a Hrp-
dependent manner.

### Example 6 -  Construction of a Minimal Hrp Secretion and Regulation System

5

The observations that DspE functions in *Pseudomonas syringae* and that
avrE functions in *Erwinia amylovora* raise the possibility that the *Erwinia amylovora*
Hrp system functions to secrete other non-*Erwinia* Avr proteins. This was addressed by
preparing *Escherichia coli* containing a minimal functional hrp gene cluster from

10    *Erwinia amylovora* strain 321 and two avirulence genes from *Pseudomonas syringae*.
The minimal hrp cluster was constructed to avoid interference by the dsp genes linked to
the hrp genes in *Erwinia amylovora* (Figure 7). A derivative of pCPP430 with the dsp
region deleted, was constructed as follows. The partition region of pCPP9 (the vector
portion of pCPP430) and the hrpN gene (from pCPP1084, Wei et al., Science 257:85-88

15    (1992), which is hereby incorporated by reference) were cloned into pBluescript II SK-
(Stratagene) in the same orientation as in pCPP430. The kanamycin resistance gene
from pHP45-Km (Fellay et al., Gene 52:147-152 (1987), which is hereby incorporated
by reference)was then inserted between the partition region and hrpN. The resulting
plasmid was transformed into *Escherichia coli* C2110 (a polAts strain, ref for Tn3HoHo)

20    containing pCPP430. ColE1 based plasmids such as pBluescript cannot replicate in
*Escherichia coli* C2110 at the nonpermissive temperature (42°C), but pSC101 based
plasmids such as pCPP9 (the vector portion of pCPP430) can replicate. The resulting
strain was grown at 42°C in medium with spectinomycin (for selection of pCPP430) and
kanamycin (for selection of the pBluescript construct). The only way that this can occur

25    is if the pBluescript construct integrates into pCPP430 by homologous recombination
between either the partition region, or the hrpN gene. When the temperature is lowered
to 30°C, a second recombination event takes place. By selecting for bacterial colonies
that are resistant to spectinomycin and kanamycin, but sensitive to ampicillin (the
selectable marker in pBluescript II SK-) it was possible to find derivatives of pCPP430

30    where the entire region of DNA between the partition region of the vector and the hrpN
gene was deleted and replaced by the kanamycin resistance gene. The resulting plasmid
was named pCPP431. It contains all of the Hrp secretion and regulatory genes, but none
of the putative avirulence genes such as dspE.

To be able to regulate the Hrp secretion system without growing the

bacteria in hrp gene-inducing minimal medium a second plasmid was constructed. The

hrpL gene is an alternate sigma factor that acts as the global regulator of the rest of the

hrp genes. hrpL was cloned into pSU21 (Bartolome et al., Gene 102:75-78 (1991),

5    which is hereby incorporated by reference) under control of the lac promoter to give

pCPP1289. When bacteria containing pCPP1289 and pCPP431 are grown in medium

containing IPTG the lac promoter is turned on resulting in production of HrpL protein

which then turns on the hrp secretion genes that form the Hrp secretion apparatus.


10   **Example 7** -   **Secretion of *Pseudomonas syringae* Avr Proteins by
                      the *Erwinia amylovora* Hrp System**

                  *Escherichia coli* DH5 containing pCPP430 and pCPP1289, or pCPP431

and pCPP1289 was used to test the new systems ability to secrete other proteins.

15   *Escherichia coli* DH5 containing pCPP430 with an insertion in hrcV was used as a

secretion-defective control. Derivatives of the avirulence genes avrB and avrPto from

*Pseudomonas syringae* were constructed in pFLAG-CTC (Sigma) so that the proteins

contained the eight-amino-acid FLAG epitope C-terminal fusions to facilitate detection

in immunoblots (Ham et al., "A Cloned *Erwinia Chrysanthemi* Hrp (type III Protein

20   Secretion) System Functions in *Escherichia Coli* to Deliver *Pseudomonas syringae* Avr

Signals to Plant Cells and to Secrete Avr Proteins in Culture," Proc. Natl. Acad. Sci.

USA, 95(17): 10206-11 (1998), which is hereby incorporated by reference). The

*Escherichia coli* strains described above were transformed with the avrB-FLAG and

avrPto-FLAG constructs. Each strain was grown in LB medium, induced with IPTG,

25   and the cultures were separated into cell and supernatant fractions. The proteins from the

supernatant were concentrated 50 fold by precipitation with 0.1 volumes of 0.15%

deoxycholate and 0.1 volumes of 100% TCA. The precipitated proteins were spun down

in a centrifuge, rinsed with acetone and dissolved in PAGE gel loading buffer. The

proteins were separated by PAGE, blotted, and the avr proteins detected with antiFLAG

30   monoclonal antibodies (Sigma). AvrB-FLAG and AvrPto-FLAG were present in the cell

and supernatant fractions from the strains containing the functional hrp gene clusters,

pCPP430 or pCPP431 (Figure 8). In contrast, the Avr proteins were present within the

cells of the strains containing the hrcV mutation in the hrp cluster, but not present in the

supernatant. Thus, the secretion is Hrp-dependent. Assay for the cytoplasmic marker (ß-

35   galactosidase revealed nearly imperceptible amounts in the supernatant fractions (not

shown). Thus, cell lysis could not account for the presence of the Avr proteins in the supernatant. Therefore, the *Erwinia amylovora* Hrp system is capable of secreting *Pseudomonas syringae* Avr proteins.

5       **Example 8 -** **Secretion of Nonbacterial Proteins**

In the Yersinia Yop secretion system the 5'-untranslated portion of the RNA encoding the secreted protein, or the N-terminal portion of the secreted protein contains the signal for secretion through the type secretion system. To test the ability of

10    the *Erwinia amylovora* Hrp secretion system to secrete nonbacterial proteins, a fusion was constructed between the 5'-end of the *Erwinia amylovora* hrpN gene and the mature portion of the human placental alkaline phosphatase gene in the vector pHG165 (Stewart et al., Plasmid 15:172-181 (1986), which is hereby incorporated by reference). The HrpN protein is the main protein secreted by the *Erwinia amylovora* Hrp secretion

15    system. The fusion construct was introduced into *Escherichia coli* DH5 (pCPP431, pCPP1289) and tested for secretion. The construct was not expressed in the *Escherichia coli* strain. The failure to secrete the fusion protein was apparently due to reasons other than the incompatibility of the HrpN secretion signal with the *Erwinia amylovora* Hrp secretion system.

20              Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose, and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.

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## WHAT IS CLAIMED:

1.      A DNA construct comprising:
a first DNA molecule encoding a functional type III secretion system;
a promoter; and
a second DNA molecule encoding a protein or polypeptide capable of being secreted by the type III secretion system, wherein the second DNA molecule is operably coupled to said promoter so that upon introduction of the DNA construct into a host cell, the encoded protein or polypeptide and the type III secretion system are expressed and the encoded protein or polypeptide is secreted.

2.      The DNA construct according to claim 1, wherein the encoded protein or polypeptide is a fusion protein comprising:
a secretion signal linked by an in-frame gene fusion to a protein or polypeptide of interest.

3.      The DNA construct according to claim 2, wherein the fusion protein further comprises:
a protease-cleavable amino acid sequence between the secretion signal and the protein or polypeptide of interest.

4.      The DNA construct according to claim 2, wherein the secretion signal is a mRNA or a polypeptide fragment of a naturally-occurring protein secreted by the type III secretion system.

5.      The DNA construct according to claim 4, wherein the secretion signal is an N-terminal polypeptide fragment of *Erwinia amylovora* DspE.

6.      The DNA construct according to claim 5, wherein the N-terminal polypeptide fragment of *Erwinia amylovora* DspE has an amino acid sequence corresponding to SEQ. ID. No. 1.

7.    The DNA construct according to claim 6, wherein the N-terminal polypeptide fragment of *Erwinia amylovora* DspE is encoded by a DNA molecule having a nucleotide sequence corresponding to SEQ. ID. No. 2.

5        8.    The DNA construct according to claim 1, wherein the first DNA molecule encodes a type III secretion system from *Erwinia amylovora* or *Erwinia chrysanthemi*.

9.    The DNA construct according to claim 1, wherein the encoded
10    protein or polypeptide is a naturally-secreted protein or polypeptide heterologous to the type III secretion system.

10.    An expression system comprising an expression vector into which is inserted a heterologous DNA construct of claim 1.
15

11.    A host cell containing a heterologous DNA construct according to claim 1.

12.    The host cell according to claim 11, wherein the host cell is
20    present in a cell culture.

13.    The host cell according to claim 12, wherein the host cell is a prokaryote.

25        14.    The host cell according to claim 13, wherein the prokaryote is *Escherichia coli*.

15.    A system comprising:
a first DNA construct comprising a first DNA molecule encoding a
30    functional type III secretion system and
a second DNA construct comprising promoter operably coupled to a second DNA molecule encoding a protein or polypeptide capable of being secreted by the type III secretion system, wherein upon introduction of the first and second DNA

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constructs into a host cell, the encoded protein or polypeptide and the type III secretion
system are expressed and the encoded protein or polypeptide is secreted.

16.    The system according to claim 15, wherein the first DNA
molecule encodes a type III secretion system from *Erwinia amylovora* or *Erwinia chrysanthemi*.

17.    The system according to claim 16, wherein the first DNA
construct is cosmid pCPP430.

18.    The system according to claim 16, wherein the first DNA
construct is cosmid pCPP2156.

19.    The system according to claim 15, wherein the encoded protein or
polypeptide is a fusion protein comprising:
           a secretion signal linked by an in-frame gene fusion to a protein or
polypeptide of interest.

20.    The system according to claim 19, wherein the fusion protein
further comprises:
           a protease-cleavable amino acid sequence between the secretion signal
and the protein or polypeptide of interest.

21.    The system according to claim 19, wherein the secretion signal is
a mRNA or a polypeptide fragment of a naturally-occurring protein secreted by a type III
secretion system.

22.    The system according to claim 21, wherein the secretion signal is
an N-terminal polypeptide fragment of *Erwinia amylovora* DspE.

23.    The system according to claim 22, wherein the N-terminal
polypeptide fragment of *Erwinia amylovora* DspE has an amino acid sequence
corresponding to SEQ. ID. No. 1.

24.     The system according to claim 23, wherein the N-terminal polypeptide fragment of *Erwinia amylovora* DspE is encoded by a DNA molecule having a nucleotide sequence corresponding to SEQ. ID. No. 2.

25.     The system according to claim 15, wherein the encoded protein or polypeptide is a naturally-secreted protein or polypeptide heterologous to the type III secretion system.

26.     A host cell containing a heterologous system according to claim 15.

27.     The host cell according to claim 26, wherein the host cell is present in a cell culture.

28.     The host cell according to claim 27, wherein the host cell is a prokaryote.

29.     The host cell according to claim 28, wherein the prokaryote is *Escherichia coli*.

30.     A method of secreting a protein or polypeptide into the environment of a host cell, said method comprising:

introducing into a host cell a DNA construct according to claim 1 under conditions effective to cause expression of the encoded protein or polypeptide and the type III secretion system, wherein the encoded protein or polypeptide is secreted by the host cell into the environment.

31.     The method according to claim 30, wherein the encoded protein or polypeptide of interest is a fusion protein comprising:

a secretion signal linked by an in-frame gene fusion to a protein or polypeptide of interest.

32.    The method according to claim 31, wherein the fusion protein further comprises:

a protease-cleavable amino acid sequence between the secretion signal and the protein or polypeptide of interest.

5

33.    The method according to claim 31, wherein the secretion signal is a mRNA or a polypeptide fragment of a naturally-occurring protein secreted by a type III secretion system.

10

34.    The method according to claim 30, wherein the first DNA molecule encodes a type III secretion system from *Erwinia amylovora* or *Erwinia chrysanthemi*.

35.    The method according to claim 30, wherein the encoded protein or

15    polypeptide is a naturally-secreted protein or polypeptide heterologous to the type III secretion system.

36.    The method according to claim 30, wherein the host cell is present in a cell culture.

20

37.    The method according to claim 36, wherein the host cell is a prokaryote.

38.    The method according to claim 38, wherein the prokaryote is

25    *Escherichia coli*.

39.    A method of secreting a protein or polypeptide into the environment of a host cell, said method comprising:

introducing into a host cell a system according to claim 15 under

30    conditions effective to cause expression of the encoded protein or polypeptide and the type III secretion system, wherein the encoded protein or polypeptide is secreted by the host cell into the environment.

40.    The method according to claim 39, wherein the encoded protein or polypeptide is a fusion protein comprising:

a secretion signal linked by an in-frame gene fusion to a protein or polypeptide of interest.

5

41.    The method according to claim 40, wherein the fusion protein further comprises:

a protease-cleavable amino acid sequence between the secretion signal and the protein or polypeptide of interest.

10

42.    The method according to claim 40, wherein the secretion signal is a mRNA or a polypeptide fragment of a naturally-occurring protein secreted by a type III secretion system.

15     43.    The method according to claim 39, wherein the first DNA molecule encodes a type III secretion system from *Erwinia amylovora* or *Erwinia chrysanthemi*.

44.    The method according to claim 43, wherein the first DNA

20    construct is cosmid pCPP430.

45.    The method according to claim 43, wherein the first DNA construct is cosmid pCPP2156.

25     46.    The method according to claim 39, wherein the encoded protein or polypeptide is a naturally-secreted protein or polypeptide heterologous to the type III secretion system.

47.    The method according to claim 39, wherein the host cell is present

30    in a cell culture.

48.    The method according to claim 47, wherein the host cell is a prokaryote.

49.     The method according to claim 48, wherein the prokaryote is
*Escherichia coli.*

5           50.     A method of isolating a protein or polypeptide, said method
comprising:

            providing a recombinant host cell comprising a first DNA molecule
encoding a functional type III secretion system and a second, heterologous DNA
molecule having a promoter operably coupled to a nucleic acid sequence encoding a
10    protein or polypeptide capable of being secreted by the type III secretion system;

            introducing the recombinant host cell into a culture medium, wherein the
encoded protein or polypeptide and the type III secretion system are expressed and the
encoded protein or polypeptide is secreted into the culture medium; and

            isolating the encoded protein or polypeptide from the culture medium.

15

            51.     The method according to claim 50, wherein the encoded protein or
polypeptide is a fusion protein comprising:

            a secretion signal linked by an in-frame gene fusion to a protein or
polypeptide of interest.

20

            52.     The method according to claim 51, wherein the fusion protein
further comprises:

            a protease-cleavable amino acid sequence between the secretion signal
and the protein or polypeptide of interest.

25

            53.     The method according to claim 52, wherein said method further
comprises:

            introducing into the culture medium, prior to said isolating the encoded
protein or polypeptide, a protease effective to cleave the protease-cleavable amino acid
30    sequence.

            54.     The method according to claim 52, wherein said isolating the
encoded protein or polypeptide comprises:

isolating the fusion protein from the culture medium;

exposing the isolated fusion protein to a protease effective to cleave the protease-cleavable amino acid sequence; and

recovering the protein or polypeptide of interest.

55.     The method according to.claim 51, wherein the secretion signal is a mRNA or a polypeptide fragment of a naturally-occurring protein secreted by a type III secretion system.

56.     The method according to claim 50, wherein the type III secretion system is a type III secretion system from *Erwinia amylovora* or *Erwinia chrysanthemi*.

57.     The method according to claim 50, wherein the first DNA molecule is heterologous to the recombinant host cell.

58.     The method according to claim 57, wherein the first DNA molecule is cosmid pCPP430.

59.     The method according to claim 57, wherein the first DNA molecule is cosmid pCPP2156.

60.     The method according to claim 50, wherein the encoded protein or polypeptide is a naturally-secreted protein or polypeptide heterologous to the type III secretion system.

61.     The method according to claim 50, wherein the host cell is selected from the group of *Escherichia coli*, *Erwinia amylovora*, and *Erwinia chrysanthemi*.

62.     A method of identifying a gene encoding a potential effector protein or polypeptide comprising:

providing a host cell comprising a DNA molecule encoding a functional type III secretion system;

introducing into the host cell a candidate gene encoding a protein or
polypeptide under conditions effective to express the encoded protein or polypeptide;
and.

determining whether the encoded protein or polypeptide is secreted by the
5    recombinant host cell, wherein secretion of the encoded protein or polypeptide indicates
that the gene encodes a potential effector protein or polypeptide.


63.      The method according to claim 62, wherein the host cell is
selected from the group of *Escherichia coli*, *Erwinia amylovora*, and *Erwinia*
10   *chrysanthemi*.


64.      The method according to claim 62, wherein the type III protein
secretion system is a type III protein secretion system of *Erwinia amylovora* or *Erwinia*
*chrysanthemi*.

15

65.      The method according to claim 62, wherein the DNA molecule is
heterologous to the host cell, said method further comprising:
introducing the DNA molecule into the host cell.


20           66.      The method according to claim 65, wherein the DNA molecule is
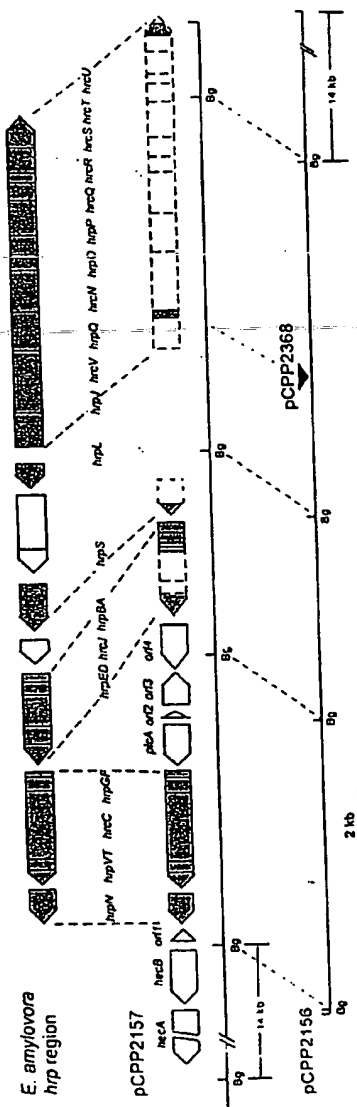cosmid pCPP430 or cosmid pCPP2156.


67.      The method according to claim 62, wherein the gene is a chimeric
gene encoding an epitope tag fused to the encoded protein or polypeptide and said
25   determining comprises:
isolating all protein or polypeptide products secreted by the host cell;
exposing the isolated protein or polypeptide products to an antibody
capable of recognizing the epitope tag; and
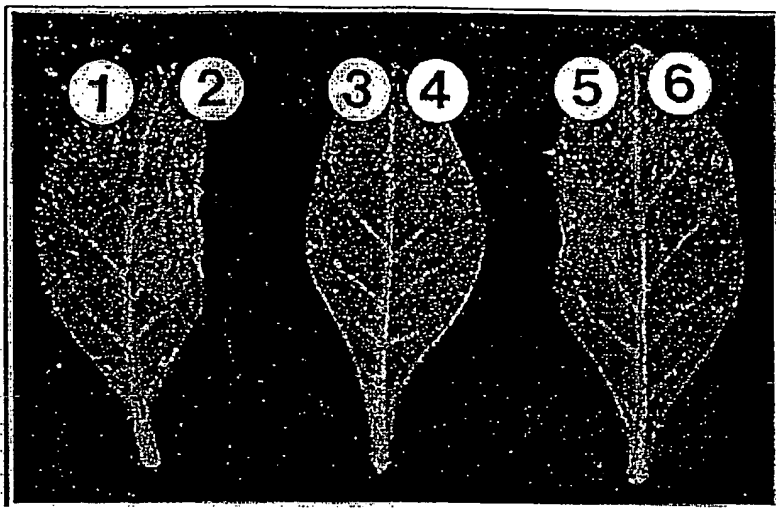detecting any antibody bound to the encoded protein or polypeptide.

30

68.      The DNA construct according to claim 4, wherein the secretion
signal is a mRNA.

69. The system according to claim 21, wherein the secretion signal is a mRNA.
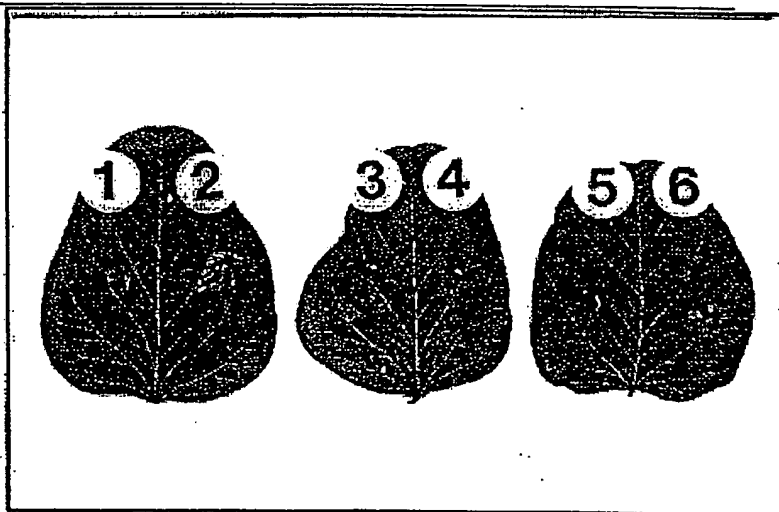
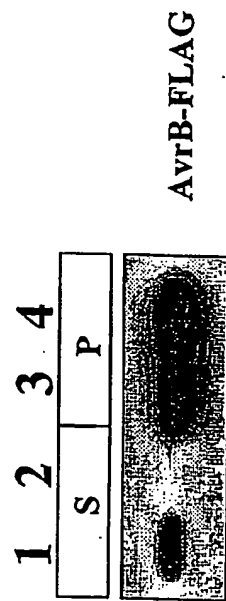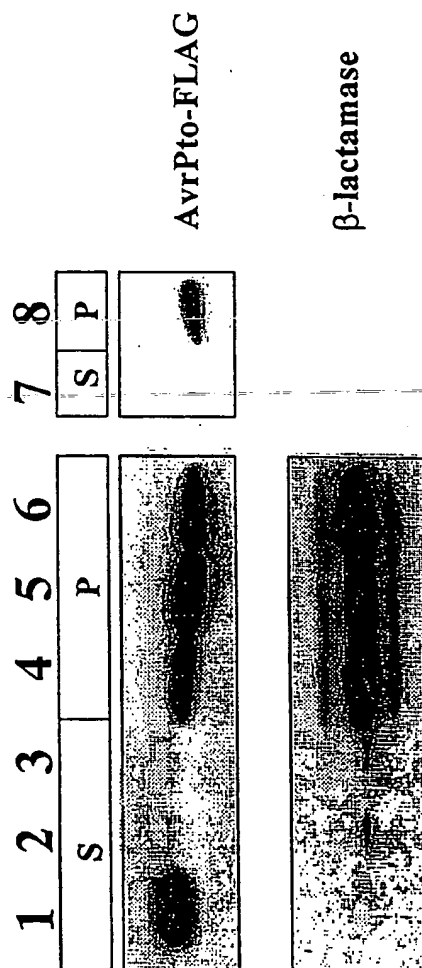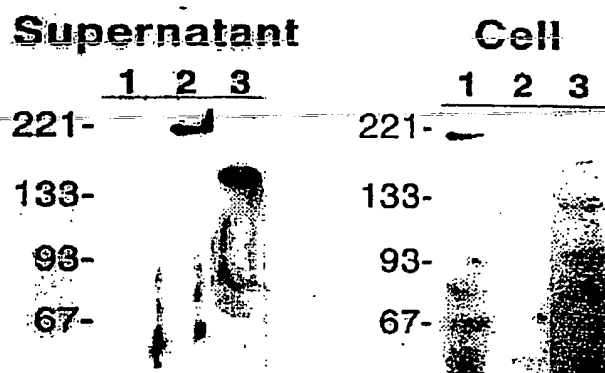FIGURE 1

A



B



FIGURE 2

FIGURE 3



AvrB-FLAG

FIGURE 4

FIGURE 5

# Immunoblots of DspE in Supernatant
# and Cell Fractions from *E. amylovora*
# Grown in HRP Inducing Medium



Lane 1. Hrp mutant Ea273-K178.
Lane 2. Wild-type strain Ea273.
Lane 3. Partial *dspE* deletion mutant
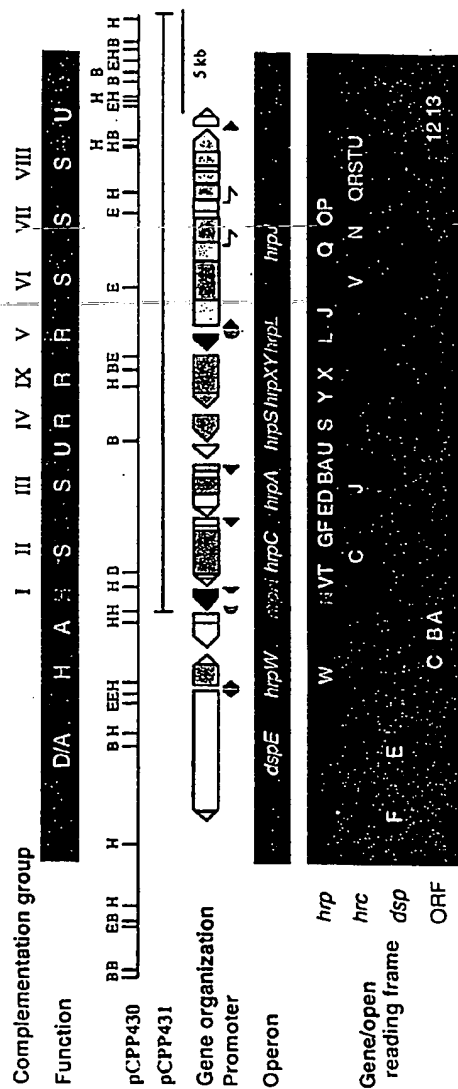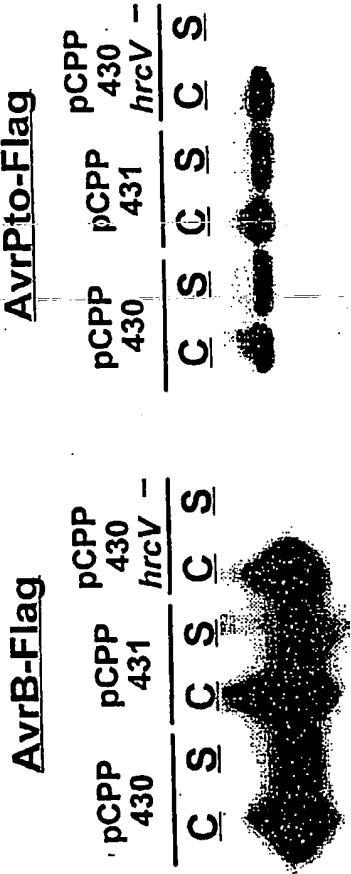strain Ea273*dspE* [2]1521.

FIGURE 6

FIGURE 7

FIGURE 8



Immunoblots of Flagged Avr proteins from Cell (C) and Supernatant (S) Fractions from E. coli DH5 with Cosmids containing Various Parts of the E. amylovora Hrp Cluster

SEQUENCE LISTING

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<120> RECOMBINANT CONSTRUCTS AND SYSTEMS FOR SECRETION OF
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<170> PatentIn Ver. 2.0

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            35              40                  45

Lys Asn Arg Gly Lys Met Pro Arg Ile His Gln Pro Ser Thr Ala Ala
        50                  55                  60

Asp Gly Ile Ser Ala Ala His Gln Gln Lys Lys Ser Phe Ser Leu Arg
 65                  70                  75                  80

Gly Cys Leu Gly Thr Lys Lys Phe Ser Arg Ser Ala Pro Gln Gly Gln
                85                  90                  95

Pro Gly Thr Thr His Ser Lys Gly Ala Thr Leu Arg Asp Leu Leu Ala
            100                 105                 110

Arg Asp Asp Gly Glu Thr Gln His Glu Ala Ala Ala Pro Asp Ala Ala
            115                 120                 125

1

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